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MOLECULARLY IMPRINTED POLYMERS SYNTHESIZED AS ADSORBENTS FOR ERGOT ALKALOIDS: CHARACTERIZATION AND *IN VITRO* AND *EX VIVO* ASSESSMENT OF EFFECTS ON ERGOT ALKALOID BIOAVAILABILITY

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DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment at the University of Kentucky

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ABSTRACT OF DISSERTATION

Alkaloid toxicities negatively impact livestock health and production and are of serious economic concern to animal industries. To date, few strategies have been developed to evaluate alkaloid levels in feed or to counteract alkaloid toxicities. The present research evaluated the applicability of imprinting technology to synthesize polymers that have potential to interact with ergot alkaloids and therefore reduce their bioavailability in the GIT. The studies also evaluated applicability of synthesized polymers for use in the ruminal environment using an *in vitro* ruminal fermentation model, and for the ability to ameliorate vasoconstriction using *ex vivo* myographic evaluations.

In the first experiment, styrene-based molecularly imprinted polymer (MIP) was synthesized using ergotamine as the imprinting template and evaluated for specificity of adsorption to various ergot alkaloids. Cross reactivity with related alkaloids exists due to similarities in structure and functional groups. Both polymers (MIP and NIP) showed strong adsorption intensity and no difference was observed for estimated maximum adsorption capacity between MIP and NIP. Morphologically, MIP was highly porous with greater surface area than NIP. Solid phase extraction indicated stronger adsorption of MIP than NIP to ergot alkaloids suggesting the potential for MIP as a sorbent material for solid phase extraction (SPE) columns used for sample clean-up prior to HPLC or LC-MS/MS analysis of complex samples.

In Experiment 2, methacrylic acid-based polymers were synthesized with ergotamine as a template. Among the 4 alkaloids evaluated for selectivity, adsorption difference between MIP and NIP interacted with alkaloid concentration, although differences were generally consistent across concentrations. Imprinting did not affect lysergol and bromocriptine adsorption, but resulted in higher adsorption to methylergonovine. However, there was no difference between MIP and NIP for adsorption of ergotamine. Hydrophobic interactions and H-bonding were the primary interactive forces between polymers and alkaloid adsorbents. Morphologically, MIP had greater surface area and porosity implying a larger surface for adsorption. In addition to its application as SPE sorbent, this MIP was a suitable candidate for application as a feed adsorbent to reduce the bioavailability of certain alkaloid in the gut.

In experiment 3, methacrylic acid-based polymers were evaluated for their effect on *in vitro* ruminal fermentation. There were no interactions between polymer type and inclusion

level, and no differences between polymer types for cumulative gas production or rate of gas production. Total gas production and rate of gas production were unaffected by inclusion level. Polymers did not affect total or individual VFA concentrations, ammonia-N or methane concentration at any inclusion level. However, a logarithmic increase in polymer dose level decreased the pH linearly with maximum depression of 0.24 units. This study indicated that, within the range of expected use levels, polymers were essentially inert and would not be expected to affect ruminal fermentation.

In experiment 4, *ex vivo* myographic bioassays were used to determine the impact of polymers on ergotamine bioavailability. Responses measured in the *ex vivo* myographic studies had similar trend as the responses generated from *in vitro* isothermal adsorption studies. Results of that study also showed that *ex vivo* myographic responses could be predicted from *in vitro* isothermal adsorption studies with more than 80% accuracy. These studies indicate that synthetic polymers are potentially effective adsorbents to mitigate ergot toxicity with little evidence of substantial differences between MIP and NIP.

KEYWORDS: ergotamine, adsorbent, myograph, imprinted polymer, isotherms, fescue

Manoj Kudupoje

Author's Signature

September 7, 2017

Date

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This dissertation is dedicated to those who have supported and encouraged me throughout my life. My parents, Bojappa and Shanthy Kudupoje, who gave me the desire to never stop learning and taught me to follow my passions. My sister Mamatha and niece Apeksha, who reminded me to take a break now and then to see where my work and dreams met the real world. Friends too numerous to name, who kept me laughing and sane through it all. But, most of all, my wife Sandhya, who married into this wild ride and helped me with every step of my life. Without her I might still be working. I am grateful for your love and support.

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FREQUENTLY USED ABBREVIATIONS

5-HT: 5-hydroxytryptamine (serotonin) receptor
ADG: average daily gain
BC: Bromocriptine
BET: Brunauer Emmett Teller
BW: body weight
CH_4: methane
CO_2: carbon dioxide
CP: crude protein
d: day
D_2: dopamine receptor 2
DE: digestible energy
DM: dry matter
DMI: dry matter intake
E-: endophyte uninfected
E+: endophyte infected
EGDMA: Ethyleneglycol Dimethacrylate
ETA: Ergotamine
FLD: Fluorescence Detector
FTIR: Fourier Transform Infrared
GC: Gas Chromatography
GIT: gastrointestinal tract
h: hour
hd: head (i.e. animal)
HPLC: High Pressure Liquid Chromatography
K: Binding Constant
K_d: Dissociation Constant
LY: Lysergol
ME: methylergonovine
min: minute
MIP: Molecularly Imprinted Polymer
MISPE: Molecularly Imprinted Solid Phase Extraction
MMA: Methyl methacrylate
MS: Mass Spectrometer
N: nitrogen
NIP: Non-molecularly imprinted polymer
O_2: oxygen
PRL: prolactin
ppb: Part Per Billion
ppm: Parts Per Million
SPE: Solid Phase Extraction
VFA: volatile fatty acid

1. INTRODUCTION

Tall fescue (*Lolium arundinaceum*) is a cool season perennial grass popularly used for forage or as pasture in livestock production (Hoveland, 2009) and is known for its drought tolerance (Richardson et al., 1999), insect resistance, and adaptability to poor soil conditions (Buckner and Bush, 1979). A primary factor that supports fescue survivability is the presence of the wild type endophytic fungus *Epichloë coenophiala* which propagates as a mutualistic symbiont (Bacon et al., 1977). An adverse relationship between endophyte infection and animal performance has been attributed to effects of secondary metabolites produced by endophytes called ergot alkaloids (Bacon et al., 1986; Lyons et al., 1990). High binding affinity between the ergot alkaloids and neurotransmitter receptors including adrenalin, serotonin, and dopamine receptors is considered as the cause for their physiological effects in livestock (Pertz and Eich, 1999). The adverse effects observed include cardiovascular changes, vasoconstriction, and reproductive problems (Thompson and Stuedemann, 1993).

Several management strategies have been in practice to minimize the toxic effects including rotational grazing, use of novel endophyte cultivars, suppression or removal of the seed head, use of dietary supplements and inter-seeding with legumes (Belesky and Hill, 1997). Recently, supplementing vasodilators including isoflavones (Aiken et al., 2016), estradiol and soyhulls have been shown to alleviate some of the toxicity symptoms. Feeding conventional toxin adsorbents has also been shown to minimize the negative effects of endophyte toxins on animal performance (Wielogórska et al., 2016). Supplementation of adsorbents in the diet has been shown to reduce the bioavailability of toxins in the gastrointestinal tract (Akay et al., 2003). However, an *in vitro* adsorption study conducted in rumen fluid demonstrated decreased adsorption affinity and capacity of several adsorbents to ergotamine tartrate (Evans and Dawson, 2000), when compared with binding in aqueous buffer. Usually decreased adsorption occurs when adsorbents bind non-specifically (Whitlow, 2006; Jard et al., 2011). Therefore, adsorbents that have good adsorption properties that can achieve specific adsorption with superior affinity and selectivity are required for adsorption in complex media. Imprinting technology is one such area where research has shown superior adsorption properties to target molecules.Manoj

Researchers have used the principles of biological interactions in imprinting technology to synthesize molecularly imprinted polymers (MIP) which have utility in applications that require high specific binding (Pauling, 1940). These biomimetic polymers have been shown to interact with target compounds with great specificity and selectivity (Mosbach et al., 2000; Pichon and Chapuis-Hugon, 2008). MIPs have been applied in different fields, especially in chromatographic separation (Sellergren et al., 1988), as a sorbent material for sample clean-up in analytical chemistry (Lanza and Sellergren, 2001; Martin-Esteban, 2001) and as a drug delivery system in biomedicine (Sellergren and Allender, 2005). Application of MIPs, especially as MISPE extraction material, have been well researched for various compounds including mycotoxins (Pascale et al., 2008), sterols (Puoci et al., 2008a), drugs (Beltran et al., 2009), amide anesthetics (Andersson et al., 2002) and organic dyes (Li et al., 2008). However, less research has been conducted on synthesis of MIPs towards ergot alkaloids or regarding application as an adsorbent in animal feed. As a feed supplement, MIPs could potentially reduce the bioavailability of ergot alkaloids and could help prevent fescue toxicosis. Additionally, MIPs could be used as an adsorbent material in solid phase extraction columns to aid in analysis of ergot alkaloids.

2. LITERATURE REVIEW

2.1. History of Fescue toxicosis

Tall fescue (*Lolium arundinaceum*) is cool season perennial grass that occupies more than 20 million hectares of pastureland in the eastern United States (Parish et al., 2003; Bacetty et al., 2009). It is popularly used for forage or as pasture in livestock production (Hoveland, 2009) and is known for its drought tolerance (Richardson et al., 1999), insect resistance, and adaptability to poor soil conditions (Buckner and Bush, 1979). A primary factor that supports fescue survivability is the presence of the wild type endophyte fungus *Epichloë coenophiala* that propagates as a mutualistic symbiont (Bacon et al., 1977). In the United States, estimates are that more than 90% of tall fescue pastures are infected with endophyte (Glenn et al., 1996), and in Kentucky the endophyte infection rate is around 85% (Lacefield et al., 2003). Despite its better yield in adverse environmental conditions, tall fescue has the reputation of causing health problems in grazing animals (Stuedemann and Hoveland, 1988) resulting in decreases in performance and economic losses in animal industries (Hoveland et al., 1983). The relationship between endophyte infection and animal performance was attributed to the adverse effects caused by secondary metabolites produced by endophytes called ergot alkaloids (Bacon et al., 1986; Lyons et al., 1990). Harmful effects in cattle were noticed when steers fed endophyte-infected fescue hay or seed exhibited toxic symptoms compared to steers fed non-infected hay or seed (Schmidt et al., 1982). Some of the adverse effects observed included cardiovascular changes, vasoconstriction, and reproductive problems (Thompson and Stuedemann, 1993). Additionally, the problems associated with fescue toxicosis were exacerbated under conditions of increased environmental temperatures (Hemken et al., 1981).

Fescue infected with endophyte contains a mixture of compounds including loline alkaloids (diazaphenanthrene and pyrrolizidine), pyrrolopyrazine alkaloids, and ergot alkaloids (Porter et al., 1977; Bush and Burrus, 1988; Clay and Cheplick, 1989; Bacon, 1995). Perloline and perlolidines are two loline alkaloids belonging to diazaphenanthrene group that have been shown to increase body temperatures in sheep (Hannah et al., 1990). The same report also attributed a decrease in apparent cellulose digestibility to these alkaloids. Pyrrolizidines, including N-acetylloline and N-formylloline (Strahan et al., 1987; Bush et al., 1993), were found to have insect repellent and insecticidal properties (Johnson et al., 1985; Dahlman et al., 1997). High concentrations of N-acetyl and N-formyl loline in tall fescue seeds have been correlated

with endophyte infection rate, and shown to suppress feed intake, and alter respiration rate and rectal temperature (Jackson et al., 1984). However, some studies indicated that loline alkaloids were only partially responsible for the fescue toxicosis syndrome since no vasoconstrictive activity in peripheral tissue (Klotz et al., 2008) and no binding affinity for D_2 receptors were observed (Larson et al., 1999). Peramine, is the only studied alkaloid in the pyrrolopyrazine group that is toxic to crop pests but is well tolerated by livestock (Easton et al., 2001; Schardl et al., 2007) with no biological activity when tested using mammalian bioassays (Bush and Fannin, 2009).

Ergot alkaloids are the most abundant and commonly encountered alkaloid compounds in tall fescue, and have been proposed to be responsible for ergotism-like toxicosis in livestock (Lyons et al., 1986). Three classes of ergot alkaloids have been identified: clavines, ergolines (lysergic acid and its derivatives) and ergopeptides. Clavine alkaloids, including chanoclavine, agroclavine and penniclavine, which are the biosynthetic precursors to lysergic acid amides and ergopeptides (Floss, 1976), have been shown to possess insecticidal properties similar to loline alkaloids (Cheplick et al., 1989), but have limited physiological effects in animals (Bacon, 1995). On the other hand, ergolines and ergopeptides are commonly found as co-contaminants in endophyte infected tall fescue. Nearly 10 to 50% of the total alkaloids in the contaminated forage are ergopeptides (Lyons, 1986). Additionally, alkaloid concentrations have been found to be higher during drought (Arechavaleta et al., 1992), warm temperatures (Belesky et al., 1987; Bush et al., 1993) and after N-fertilization of plants (Lyons and Bacon, 1984; Lyons, 1986). Ergovaline is the predominant ergot alkaloid, comprising nearly 84 to 97% of the ergopeptide alkaloids in tall fescue infected with *Epichloë* (Lyons, 1986; Bush et al., 1997), but effects of fescue toxicosis may be attributed to additive effects of numerous alkaloids (Klotz et al., 2008).

2.2. Ergot alkaloids: Structure

The biosynthetic pathway of ergot alkaloids in endophytic fungi begins with a rate limiting reaction between dimethylallyl diphosphate (DMAPP, a melvonic acid derivative) and L-tryptophan, catalyzed by DMAT synthase, forming dimethylallyl-tryptophan (DMAT) (Gebler and Poulter, 1992). Through several steps involving α-N-methylation and oxidation, DMAT is cyclized (Schardl and Panaccione, 2005; Potter et al., 2008), and converted to lysergic acid and its derivatives with a characteristic heterogeneous tetracyclic ring structure called ergoline (Berde, 1979; Weber et al., 1980).

Ergolines contain the lysergic ring structure with hydroxyl, carboxyl, or carboxamide functional groups (Figure 2-1) (Hill et al., 2001). Epimeric forms (R or S form) of ergot alkaloids exist due to the stereogenic center at the C-8 position of ergoline (Berde and Stürmer, 1978). On the other hand, ergopeptides are derivatives of lysergic acid that are formed by condensation with three amino acids forming a cyclol moiety attached at the carboxamide site of the basic ergoline ring (Figure 2-2) (Keller, 1999; Panaccione and Schardl, 2003). The unique feature of ergopeptides is the cyclol bond (-N-C(OH)-) found at the juncture between the two cyclic amides. While all ergopeptides have proline as a common amino acid, the other two amino acids involved in the formation of the cyclic tripeptide vary (Panaccione and Schardl, 2003). Variation between ergopeptides occurs at the R1 and R2 substituents of the tripeptide cyclol moiety (Figure 2-2) (Schardl and Panaccione, 2005).

2.3. Toxicodynamics

The structural similarities between the ergot alkaloids and particular neurotransmitters is considered as the cause for their physiological effects in livestock. These compounds are known to have high binding affinity towards either adrenergic, serotonergic, or dopaminergic receptors (Pertz and Eich, 1999). The intensity of physiological effects depend on tissue type, number and type of receptors on the tissue and interaction affinity (Berde and Stürmer, 1978). These researchers also showed that alkaloids can act as agonists or antagonists on receptor sites of biogenic amides. There is on-going debate regarding the potency of different ergot alkaloids in producing biological effects. A dose–response study performed in broiler chickens to identify the lowest and no observed adverse effect levels (LOAEL and NOAEL, respectively) showed that toxicity was time-dependent (Dänicke, 2017). While LOAEL corresponded to a total dietary ergot alkaloid (sum of ergometrine, ergocornine, ergotamine, ergocryptine, ergosine, ergocristine and their -inine isomers) content of 5.7 mg/kg until day 14 of age, it decreased to 2.03 mg/kg when birds were exposed for a period of 35 days. Consequently, NOAEL corresponded to a total alkaloid content of 2.49 mg/kg diet until day 14 of age, while 1.94 mg/kg diet applied until day 35 of age. There is no regulatory limit defined for ergot alkaloids in the USA. However, the current feed safety regulations as compiled by European food safety authority (EFSA) specify an upper limit of 1000 mg ergot (*C. purpurea*) per kg unground cereal grains (Directive, 2002) but does not consider the ergot alkaloid concentrations and its variations in ergot. Information on toxicity data for a few ergot alkaloids in rabbits (Griffith et al., 1978), rat and mouse are

established (Rothlin, 1947). Lethal dose (LD_{50}) of some ergot alkaloids in lab animals after intravenous (i.v.) or oral dose (Rutschmann et al., 1978) is presented in table 2-1.

Even though recent studies have implicated ergopeptides as the main etiological agent of fescue toxicosis, research conducted by Schiff (2006) indicated a strong physiological response to ergonovine, a lysergic acid amide. Ergonovine caused direct stimulation of uterine smooth musculature resulting in increased muscular tone and in the rate and force of rhythmical contractions. On the contrary, two ergoline alkaloids, ergonovine and ergine had lower affinities comparted to ergopeptides including ergovaline, ergotamine, and ergocryptine (Larson et al., 1995; Larson et al., 1999). Additionally, lysergic acid did not induce significant vasoconstriction in bovine right ruminal artery and vein (Foote et al., 2011a) or in bovine lateral saphenous vein (Klotz et al., 2006). Foote (2011) showed that arterial smooth muscle contraction was induced at concentration of 10^{-6} mol for ergovaline and ergotamine; at 10^{-5} mol for ergocryptine, ergocornine, and ergonovine; and at 10^{-4} mol for ergocristine, suggesting that ergovaline and ergotamine can induce toxicity at comparatively low concentrations. Ergovaline has been implicated as the major alkaloid in fescue toxicosis (Yates et al., 1985; Hoveland, 1993). Ergovaline induced vasoconstriction of smooth muscles was implicated in reduced blood flow to peripheral tissue leading to reduced peripheral skin temperature (McLeay et al., 2002). In contrast to ergovaline, ergotamine is usually present at much lower concentration in contaminated fescue (Yates et al., 1985), but is considered to have a similar type of physiological effect, i.e., agonistic activity on serotonin receptors causing vasoconstriction (Silberstein and McCrory, 2003). Even though ergopeptides are often implicated as the causative agent of fescue toxicity, it is likely that the observed symptoms are caused by an additive or synergistic mechanism of several alkaloids.

2.3.1.Altered hormones

Ergot alkaloid-induced altered thermoregulation and increased smooth muscle contractile activity of blood vessels was discovered as early as 1973 (Floss et al., 1973). That study also revealed altered cardiovascular function, gastrointestinal motility and endocrine function. Cattle consuming ergotamine exhibited changes in plasma concentration of metabolic hormones including increases in thyroid hormones and reduction in insulin (Browning, 2000). Research conducted by the same authors also indicated altered reproductive hormones including reductions in luteinizing hormone and increases in prostaglandin F2-α (Browning et al.,

2001). Similarly, ergovaline has also had inhibitory effects on reproductive hormones such as prolactin and progesterone (Brendemuehl et al., 1994; Redmond et al., 1994) causing prolonged gestation, spontaneous abortion, placental abnormalities, and dystocia (Cross et al., 1995; Rohrbach et al., 1995). Additionally, ergopeptides have been shown to be potent inhibitors of bovine vesicular glutamate transporters (VGLUT) activity compared to ergolines (Carlson et al., 1989). Ergopeptides including ergotamine, ergovaline and bromocriptine decreased VGLUT activity by 80-90% (Xue et al., 2011), whereas the ergolines including ergonovine and lysergic acid showed little to no inhibition on VGLUT activity. Furthermore, earlier studies showed interactions between alkaloids and neuro-hormone receptors, including adrenaline, dopamine and serotonin receptors, and therefore attributed the cause of physiological effects in animals to stimulation of neuro-transmitter receptors (Oliver et al., 1993; Thompson and Stuedemann, 1993). Even though factors like physiological stress, α-adrenergic agonism and dopamine receptor agonism can alter growth hormone secretion (Greenspan et al., 2004), studies could not demonstrate any differences in levels of circulating growth hormone (Elsasser and Bolt, 1987; Lipham et al., 1989).

2.3.2.Adrenergic effects

Alpha-2 adrenergic receptors are present in bovine lateral saphenous veins and bovine dorsal pedal veins (Solomons et al., 1989) and when stimulated can induce a vasoconstrictive response (Oliver et al., 1998). Research has shown that ergot alkaloids induced stronger α-2 adrenergic agonistic activity compared to the activity on α-1 receptors (McPherson and Beart, 1983). Comparing α-1 and α-2 adrenergic agonists on contractile response of saphenous vein from animals grazing endophyte infected (E+) and non-infected (E-) pasture, there was no difference in contractile response to α-1 agonist (phenylephrine), but there was a higher contractile response to an α-2 agonist (BHT-920) in animals grazing E+ pastures, indicating greater responsiveness of α-2 adrenergic receptor in E+ animals (Oliver et al., 1993).

2.3.3.Dopaminergic effects

Studies have also shown that the binding affinities of ergopeptide and ergoline alkaloids as agonists for the D_2-dopamine receptors were similar to that of dopamine (Larson et al., 1995; Larson et al., 1999). Comparing the affinities of alkaloids to dopamine receptors, ergopeptides (ergovaline, ergotamine, and ergocryptine) displayed greater binding affinities than two ergoline

alkaloids (ergonovine and ergine) (Larson et al., 1999). Stimulation of D_2-dopamine receptors has been shown to reduce or inhibit vasoactive intestinal peptide-stimulated production of cyclic AMP, thus affecting signal transduction pathways and cellular function (Larson et al., 1995). Additionally, D2- receptor stimulation can suppress the release of prolactin (PRL), which regulates milk synthesis and secretion, thus negatively affecting lactation (Berde and Stürmer, 1978; Goldstein et al., 1980; Goetsch et al., 1987). Ergopeptides, including ergocornine, ergocryptine and 2-bromo-α-ergocryptine were found to interact with D_2 dopaminergic receptors in cattle (Lu et al., 1971; Fiore et al., 1987), thus inhibiting PRL release from the anterior pituitary. Cattle on E+ fescue pastures show significant reduction in circulating PRL and milk production (Schillo et al., 1988). Expression of genes associated with prolactin secretion was also down regulated in cultured pituitary cells when treated with ergocryptine (Maurer, 1981). It was suggested that variables relating to these effects are influenced by the agent, dosage, species, tissue, endocrinological and physiological state, as well as prior exposure to endophyte infected tall fescue and experimental conditions (Schiff, 2006).

2.3.4.Serotonergic effects

Serotonin interacts with the satiety center in the brain and agonistic activity could cause a reduction in feed intake (Simansky, 1995). Ergot alkaloids act as agonists on serotonergic (5-HT) receptors (Dyer, 1993), which was confirmed by reversal of its activity using a serotonergic receptor antagonist: metoclopramide (Lipham et al., 1989). Stimulation of serotonergic receptors have been shown to alter gut motility (Talley, 1992) and to affect the thermo-regulatory center in the hypothalamus (Lin et al., 1983; Gudelsky et al., 1986). Additionally, vasoactivity effects through persistent and irreversible interaction between ergovaline and 5-HT2A and 5-HT1B/1D receptors has been noticed in rat tail and guinea pig arteries (Schöning et al., 2001). Blocking 5-HT2A receptors of uterine and umbilical arteries using ketanserin greatly reduced the contractile response produced by ergovaline (Dyer, 1993). Furthermore, ergopeptides produced stronger agonistic responses on 5-HT2 and 5-HT2A compared to 5-HT2B, 5-HT1B, and 5-HT7 receptors of the lateral saphenous vein in cattle (Klotz et al., 2012). 5-HT2 receptor agonisms are known to cause hyperthermia while 5-HT1A agonism can induce hypothermia (Gudelsky et al., 1986). Stimulation of the hypothalamic thermal regulatory center via ergot alkaloids may alter body temperature, apart from the mechanism of reduced blood flow due to vasoconstriction.

2.4. Ergokinetics

In ruminants, ergot alkaloids consumed in the diet are exposed to pregastric rumen microbial fermentation. A decrease in the toxicity of contaminated diet in Harlan Sprague-Dawley rats was noticed when endophyte infected seeds were exposed to rumen fluid for 24h, suggesting inactivation or degradation of ergot alkaloids (Westendorf et al., 1992). However, other studies have shown that lysergic acid (Ayers et al., 2009) and ergovaline (Moyer et al., 1993) concentrations increased when contaminated diet was added to rumen inoculum *in vitro*, suggesting the release of ergot alkaloids from plant tissue during microbial fermentation. Studies have also suggested that comparatively long retention times of digesta in the gastro intestinal tract of ruminants may increase release of alkaloids from the feed and facilitate transformation to other forms of alkaloids (Hill, 2005; Ayers et al., 2009). Evaluating the recovery profile of ergot alkaloids in the excretions (feces and urine) of crossbred wethers fed tall fescue straw, nearly 35 and 248% of dietary concentrations of ergovaline and lysergic acid were recovered, respectively, suggesting likely conversion of ergovaline to lysergic acid (De Lorme et al., 2007). Additionally, studies have shown an increase in certain alkaloids in the urine or feces compared to dietary concentrations and postulated the conversion of ergot alkaloids either in the rumen before absorption or as a consequence of hepatic metabolism after absorption or subsequent to release from the subcutaneous fat that has been shown as a site of bioaccumulation (Realini et al., 2005; Klotz et al., 2009).

Several studies have identified adsorption sites for ergot alkaloids in the gastrointestinal tract and have suggested the mechanism of transport. In ruminants, nearly 40-50% of the total dietary ergot alkaloids are either transformed or absorbed through the rumen (Westendorf et al., 1992; Hill et al., 2001) and the remaining quantity (50-60%) passes on to abomasum. Westendorf et al. (1992) also showed that only 5% of the total alkaloid intake was excreted in feces suggesting a total absorption of 93-97%. Several ergot alkaloids including lysergic acid, lysergol, ergonovine, ergotamine, and ergocryptine have been shown to cross parabiotic membranes prepared from ruminal, reticular, and omasal tissues of sheep gut (Hill et al., 2001). Among the tissues, the ruminal layer showed greater absorption potential than the reticular layer for total alkaloids and omasal tissue exhibited more absorption potential to ergopeptides compared to ruminal tissue. For ergovaline, conflicting results in absorption profiles have been reported, where no detectable absorption through ruminal, reticular, and omasal membranes of

sheep gut (Ayers et al., 2009), was contradicted by a flux across ruminal epithelium using a Ussing chamber (Foote et al., 2013). Studies conducted using Caco-2 cells (human intestinal cells) have shown an absorption rate of 7.5 ng/cm^2·min for ergovaline/ergovalinine (Shappell and Smith, 2005). Also, the involvement of the active transport mechanism for the ionic form of alkaloids (e.g., lysergic acid) (Hill et al., 2001) and the possibility of passive transport for basic alkaloids that exist in both ionic and non-ionic form in equimolar ratios in the pH range of intestinal lumen (Hill, 2005) have been suggested. Additionally, molecular weight and solubility in the intestinal tract affects absorption of ergot alkaloids. Ergolines were found to be transported at 20-times higher concentrations in blood compared to ergopeptides, owing in part to lower absorption of ergopeptides from the GIT due to their hydrophilic and lipophilic properties (Eckert et al., 1978). Furthermore, ergopeptides are larger molecules (>450Da) and are less soluble in GI contents compared to ergolines (<350Da). Due to hydrophilic nature, ergolines are more readily excreted in urine, while higher proportions of ergopeptides are excreted in bile owing to its lipophilic nature (Eckert et al., 1978; Hill, 2005).

Very few studies have been conducted to determine the fate of ergot alkaloids after absorption. Studies in ruminants have shown that ergot alkaloids altered enzyme profiles along with expression of genes related to detoxification in hepatic tissues (Settivari et al., 2006; Brown et al., 2009). Additionally, some studies have established that ergot alkaloids are metabolized by cytochrome P450s, specifically CYP3A, in rats (Peyronneau et al., 1994), humans (Ball et al., 1992) and ruminants (Moubarak and Rosenkrans, 2000). Ergotamine and its isomer were converted to a hydrophilic metabolite by beef liver microsomes in a time dependent fashion in the presence of the NADPH generating system (Settivari et al., 2006). A high ratio of circulating:consumed ergoline alkaloids was suggested to be a consequence of either conversion of ergopeptide to ergolines in the rumen prior to absorption or in the liver before excretion (Hill et al., 2001). Furthermore, ergot alkaloids were detected in the adipose tissue of animals that were finished on endophyte-infected tall fescue, indicating bioaccumulation in grazing animals (Realini et al., 2005). Bioaccumulation of ergovaline in the veins and arteries of animals leading to persistent contractile response of vasculature, especially uterine and umbilical arteries (Dyer, 1993) and lateral saphenous veins (Klotz et al., 2007) has been noticed while studying the *ex vivo* contractile response of ergot alkaloids.

Studies conducted to evaluate the excretion profile of ergot alkaloids in ruminants showed that the majority of alkaloids are excreted in urine (94%) and the remaining 6% in bile (Stuedemann and Hoveland, 1988; Stuedemann et al., 1998). Stuedemann et al., (1998) also showed that the first appearance of ergot alkaloids in urine occurred around 12 h after initial endophyte-infected pasture consumption and that detection persisted for 96 h after withdrawal of animals from endophyte-infected pasture. In another study, nearly 55% of the lysergic acid was excreted in urine, but ergovaline was not detectable in urine (DeLorme et al., 2007). A study conducted in non-ruminants showed excretion of ergopeptide through bile, and of ergolines via urine (Nimmerfall and Rosenthaler, 1976). Even though studies have shown the excretion profile for a few alkaloids, more studies are required to understand excretion of the wide variety of alkaloids that exist as co-contaminants.

2.5. Signs of Fescue toxicosis in animals

In the 1950s, researchers believed that a toxic substance in tall fescue caused constriction in peripheral vascular tissues and post mortem analysis of cattle consuming tall fescue hay showed thrombosis of the arteries (Jensen et al., 1956). Clinical signs including edema, hemorrhage, and thrombi in blood vessel of the limbs were noticed in sheep consuming tall fescue (Garner and Cornell, 1978). In the 1970s, an endophytic fungus *Epichloë typhina* was isolated from the tall fescue (Bacon et al., 1977), leading to the proposal of endophytic fungal toxins as the causative agent of fescue related animal health problems. In late 1980s, Solomons et al. (1989) demonstrated strong vasoconstrictive properties of the endophytic toxin, ergotamine, on bovine dorsal pedal veins. In the 1990's, it was confirmed that ergopeptides have strong vasoconstrictor properties, especially on bovine blood vessels like uterine and umbilical arteries (Dyer, 1993). Ergolines, including lysergic acid and its analog lysergic acid amide, have been shown to cause constriction of bovine lateral saphenous vein (Oliver et al., 1993; Klotz et al., 2006). Conversely, the loline alkaloid, N-acetyl loline, induced minimal constriction in venous blood vessel and no constriction in arterial vasculature of horses or cattle (Abney et al., 1993; Klotz et al., 2008). Some researchers suggested that veins are more sensitive than arteries to ergot alkaloids due to greater interaction between the alkaloids and α-2 adrenergic receptors, the dominant receptors in veins (Oliver et al., 1993; Oliver et al., 1998). Angiotensin converting enzyme concentrations were higher in cattle grazing endophyte infected tall fescue (Oliver, 1997), and endogenous nitric oxide was lower in vascular epithelium of rats fed fescue seed

contaminated with ergot alkaloids (Al-Tamimi et al., 2007) indicating altered parameters that are responsible for vasoconstriction. After several years of research, the problems associated with fescue toxicosis was classified into four major categories based on observable symptoms and physiological changes: summer slump, fescue foot, fat necrosis and reproductive effects.

2.5.1.Summer slump

Summer slump (summer syndrome) is the most prevalent fescue toxicosis effect on performance parameters in livestock production (Schmidt and Osborn, 1993; Spiers et al., 2005b). Animals grazing fescue pastures exhibited nearly a 45g/d reduction in weight gain for every 10% increase in infection rate of endophyte in the fescue (Stuedemann and Hoveland, 1988; Crawford et al., 1989). The symptoms were more prevalent during hot weather, characterized by increased respiration rate and salivation (Wagner, 2008), rough hair coats, elevated rectal temperature and animals seeking shade or wet spots (Thompson and Stuedemann, 1993). The outward symptoms of summer slump suggest that the animals were unable to dissipate heat adequately causing an increase in respiration rate to avoid hypoxemia (Oliver, 1997). Additionally, rough hair coat occurred due to retention of winter hair coat in the spring (Schmidt and Osborn, 1993).

Traditionally, several of the fescue toxicosis symptoms have been believed to be caused by altered thermoregulation due to vasoconstriction and related reduction in blood flow (Solomons et al., 1989; Rhodes et al., 1991; Klotz et al., 2006). A decrease in the skin temperature due to lowered blood flow to the subcutaneous areas and extremities (Dyer, 1993; Oliver, 1997) and or increase in core body temperature due to reduced heat loss (Neal and Schmidt, 1985; Aldrich et al., 1993) has been established. A 50% reduction in blood flow to the skin over the ribs and a decrease in the blood flow to internal organs including kidney, duodenum and colon have been established in steers consuming endophyte-infected tall fescue seed, using radiolabeled microspheres (Rhodes et al., 1991). Vasoconstriction of the caudal artery was observed using doppler ultrasonography in heifers fed endophyte-infected tall fescue seed (Aiken et al., 2007). In a subsequent study, a persistent reduction in luminal area of caudal arteries after 51 h of ergovaline consumption was observed (Aiken et al., 2009). Additionally, apart from vaso-constriction, ergot alkaloids have been shown to cause morphological changes in vasculature including thickening of small peripheral blood vessels (Garner and Cornell, 1978) and a decrease in inner diameter of blood vessels (Rhodes et al., 1991; Klotz et al., 2012) that could inhibit

blood flow. Most studies suggested that a reduction in blood flow to the skin would reduce evaporative heat loss thus making animals more susceptible to heat stress while a reduction in blood flow to the extremities during cold season would lead to hypothermia or gangrene of the extremities.

During the summer season, cattle consuming endophyte infected tall fescue have exhibited decreased feed intake, decreased average daily gain, reduced conception rate and lower milk production (Bush et al., 1979; Hemken et al., 1981; Hoveland et al., 1983; Aldrich et al., 1993). Behavioral changes including increased time standing in water or seeking shade and wallowing in mud were also noticed. Intake reductions of 10-50% have been suggested to be primarily due to the increased body temperature (Bond et al., 1984). Additionally, in cow-calf pairs on endophyte infected pastures, cows had reduced milk production and decreased intake and calves had a significantly lower weaning weight (Paterson et al., 1995), and lower average daily gain (Newsome et al., 1989; Schmidt and Osborn, 1993). As a behavioral change in steers on endophyte infected tall fescue, studies have noted that animals preferred areas dominated by clover rather than fescue in pastures (Garner and Cornell, 1987), and a tendency of animals to graze at night (Stuedemann et al., 1985).

2.5.2.Fescue foot

Cattle displaying fescue foot generally first present with a reduced weight gain or weight loss (Waller, 2009). Fescue foot can be attributed to constriction of the peripheral vasculature by ergot alkaloids, especially during the cold environmental temperatures of winter (Garner and Cornell, 1978). Alterations in the blood flow to the extremities (Bony et al., 2001; McLeay et al., 2002), most often in the hind legs (Thompson and Stuedemann, 1993) was observed in animals consuming E+ diets. Some of the important signs of fescue foot included inflammation, edema, dry gangrene and necrosis of the tissue (Bush et al., 1979), hyperemia of the hoof coronary band, damage to cells lining blood vessels and enhanced blood clotting within one to three weeks after introducing animals to E+ fescue (Oliver, 1997). Additionally, studies have shown that the ergot alkaloids can stimulate the α-2 adrenergic receptors on the blood platelets which may increase the production of thromboxane leading to platelet aggregation and thrombosis (Oliver, 1997). In severe cases of fescue foot, symptoms including swollen arteriole walls, thrombi in the blood vessels, reduced lumen size (Garner and Cornell, 1978), swelling of the fetlock, lameness, dead tissue (Schmidt and Osborn, 1993; Waller, 2009), sloughing of the hoof

(Jacobson et al., 1963), and loss of the limb between the hoof and dewclaw (Hemken et al., 1984) have been noticed.

2.5.3.Fat necrosis

Another symptom commonly associated with fescue toxicosis in grazing cattle is the necrosis of fat, especially the mesenteric fat, which is also known as lipomatosis (Wilkinson et al., 1983). This condition is characterized by the presence of hard fat masses in the mesenteric adipose tissue of the abdominal cavity along the gastrointestinal tract and is most often seen when high levels of nitrogen fertilizers are used (Stuedemann et al., 1985). The necrotic fat deposits can cause strangulation of the intestines and hinder normal flow of digesta through the GIT (Schmidt and Osborn, 1993). Fat deposits were also suggested to cause reproductive issues like dystocia (Wilkinson et al., 1983; Thompson and Stuedemann, 1993). Additionally, necrotic fat can become hard when encapsulated with fibrous connective tissue (Rumsey et al., 1979) and can undergo necrotic steatitis and saponification (Smith et al., 2004). Compared with normal fat, a higher percentage of saturated fatty acids (Townsend et al., 1991; Realini et al., 2005), protein, ash and cholesterol (Bush et al., 1979) was noticed in animals consuming E+ diets. Additionally, histological changes in fat cells including dissolution of nuclei and basophilic granular filling was observed (Smith et al., 2004) which may explain altered fat metabolism in adipocytes.

2.5.4.Reproductive effects

Heifers consuming infected fescue had reduced calving rates (Porter and Thompson, 1992), reduced conception rates (Burke et al., 2001), reduced birth weight of calves (Watson et al., 2004) and higher embryonic mortality (McEvoy et al., 2001). Depressed serum prolactin levels associated with a nearly 20-50% decrease in milk production has also been shown in cows grazing infected fescue compared with cows grazing orchard grass (Peters et al., 1992; Brown et al., 1996). Progesterone levels were lower in weanling heifers grazing infected fescue which was suggested to be a reason for reduced corpus luteum diameter, reduced size of the dominant follicle, fewer large follicles and abnormal corpus luteal function (Mahmood et al., 1994; Burke et al., 2001). Additionally, higher concentrations of mitochondria, lipid droplets and secretory granules were observed in corpus lutea of heifers grazing endophyte infected fescue (Ahmed, 1990). Heifers on high endophyte fescue pasture exhibited less estrous activity indicating luteal

dysfunction (Mahmood et al., 1994) and the follicular changes occurred independent of circulating plasma FSH levels (Browning et al., 1998). Cows injected with ergonovine and ergotamine had lowered plasma luteinizing hormone concentration and increased prostaglandin F2-α metabolite secretion (Browning et al., 1998) confirming the luteal dysfunction and reproductive problems caused by ergot alkaloids.

2.5.5.Blood parameters

Studies have shown that animals on infected tall fescue have reduced counts of neutrophils, lymphocytes, eosinophils, and monocytes (Oliver, 1997), reduced monocyte but increased eosinophils (Brown et al., 2009), decreased eosinophils and hemoglobin but increased total erythrocyte (Oliver et al., 2000) and reduced hemotocrit value (Thompson and Stuedemann, 1993). Hemotocrit value could be affected by reduced serum prolactin that is generally correlated to reduced RBC production (Socolovsky et al., 1998). Serum cholesterol levels were decreased when animals were exposed to ergot alkaloids (Stuedemann et al., 1985; Cincotta and Meier, 1989) indicating an increase in the stress levels and altered lipid metabolism (Realini et al., 2005). Additionally, an increase in serum free fatty acids levels were noticed in cows grazing endophyte-infected fescue (Peters et al., 1992). Copper levels in the blood and liver (Bonham et al., 2002) and serum prolactin (Rice et al., 1997) are known to influence humoral antibody synthesis. Animals on endophyte infected tall fescue showed higher levels of adverse effects to lipopolysaccharide challenge and had lower antibody response to vaccination (Filipov et al., 1998) with lower alpha and gamma immunoglobulins (Oliver et al., 2000) suggesting that animals on E+ diet are immunocompromised. Researchers have also suggested that the compromise in immune response could be due to reduced protein and energy intake (Galyean et al., 1999), or low prolactin (Rice et al., 1997) and copper level (Stabel et al., 1993) that are usually observed in animals on endophyte infected pasture.

2.5.6.Energy metabolism

The energy expenditure to maintain thermoregulatory activities of the animals exposed to extreme environmental temperatures which is aggravated by ergot alkaloids may partially account for the reduction in growth of cattle on endophyte infected fescue pasture (Stanier et al., 1984). Rats injected with ergovaline exhibited severe reductions in metabolic rate during heat or cold stress compared to thermoneutral temperatures (Spiers et al., 1995; Zhang et al.,

2005). Additionally, sheep dosed with plant phenolic compounds had increased whole body energy expenditure and increased urinary energy output (Iason and Murray, 1996). Animals consuming endophyte infected fescue had reduced feed intake indicating animals in negative energy balance (Bond et al., 1984) impacting production parameters including body weight gain, milk production and reproductive efficiency (Wagner, 2008).

Researchers have shown altered hepatic function in steers grazing high endophyte pastures (Piper et al., 1991; Oliver, 1997). Rats fed E+ diets (ad libitum) had low glycogen stores in the liver and exhibited upregulation of genes responsible for gluconeogenesis (PEPCK) (Settivari et al., 2006). Additionally, steers grazing high endophyte pastures also had increased levels of PEPCK-C indicating an increased gluconeogenesis (Brown et al., 2009). In the same study, animals at slaughter had reduced lactose dehydrogenase (LDH), increased aspartate amino transferase (AST) and reduced liver weights (10%) when compared to steers grazing low endophyte pastures. Low LDH suggests a decreased ability to inter-convert muscle sugar source (pyruvate and lactate) for gluconeogenesis and increased AST implies an increased ability for the interconversion of aspartate and oxaloacetate (OAA) in the TCA cycle and subsequently phosphoenolpyruvate (PEP). Ergotamine injected intravenously elevated blood glucose and reduced circulating insulin levels (Browning et al., 1997) supporting the results from other research, mainly the upregulation of gluconeogenesis in animals consuming endophyte-infected fescue.

Additionally, some studies have shown that ergot alkaloids induce vasoconstriction of blood vessels in the bovine foregut, thus reducing blood flow to the absorptive surface where approximately 45% of the digestible energy is absorbed (Kristensen and Harmon, 2005; Foote et al., 2013). A reduction in blood flow to the rumen could reduce nutrient absorption leading to reduced growth rate and general unthriftiness. Cattle consuming endophyte-infected tall fescue forage or seeds showed decreased ruminoreticular, duodenal and colonic blood flow (Rhodes et al., 1991; Foote et al., 2013), and decreased net portal flux for acetate and absorption of volatile fatty acid from the reticulorumen (Harmon et al., 1991) suggesting altered nutrient absorption from gastro intestinal tract. Additionally, intravenous injection of ergotamine and ergovaline in sheep have been shown to increase baseline tone of the reticulorumen smooth muscles and decrease the forestomach motility (McLeay and Smith, 2006; Poole et al., 2009). Dosing endophyte-infected tall fescue seed into rumen decreased particulate passage rate, liquid flow

rate and forestomach motility (Foote et al., 2013; Koontz et al., 2014). Furthermore, ergot alkaloids could also negatively impact the rumen epithelium directly by altering the absorptive and barrier functions of the epithelium (Foote et al., 2013).

2.6. Mitigation strategies to reduce ergot alkaloids toxicity

2.6.1.Management approach

Several management practices have been used to minimize the toxic effects of ergot alkaloids including rotational grazing, use of novel endophyte cultivars (E- diet), suppression or removal of the seed head, provision of mineral supplements and inter-seeding with legumes. Intensive grazing with careful attention to fertilization and weed control have been shown to reduce ergot alkaloid concentration in vegetative tillers and permit allocation of carbohydrates toward plant regrowth instead of alkaloid production (Belesky and Hill, 1997). Greater concentrations of alkaloids are present in leaf sheath than in blades (Rottinghaus et al., 1991), so adopting practices that maximize consumption of leaf blades (i.e. rotational grazing) was recommended. Another approach for reducing toxicosis is by developing tall fescue cultivars artificially infected with novel endophyte strains that do not produce toxic ergot alkaloids. Studies using the novel endophyte cultivars have been shown to increase average daily gain (ADG) and lower body temperature (Nihsen et al., 2004). Novel endophyte tall fescue plantings may resist re-colonization or infection by toxic-endophyte tall fescue from the soil seed bank (Barker et al., 2005). This particular novel endophyte fescue is known to have low carrying capacity (Gunter and Beck, 2004), but animals consuming E- grass had overall greater body weight gain per acre than those consuming E+ grass (Hancock and Andrae, 2009). Studies have also shown that endophyte infection and ergot alkaloid levels are usually highest in the seed head followed by stems in less utilized fescue grass. (Rottinghaus et al., 1991; Aiken et al., 2012). Some authors have suggested reduction of seed-head formation with herbicides (mefluidide) (Moyer and Kelley, 1995; Paterson et al., 1995) to mitigate fescue toxicosis. In one study, steers grazing endophyte-infected tall fescue that was treated with herbicide (Chaparral), had greater ADG, higher serum prolactin levels and lower rectal temperatures than steers on untreated fescue (Aiken et al., 2012).

One recommended management practice is moving cattle to warm season grass pastures during the late spring and early summer when alkaloid concentrations and seed head

formation are at peak (Forcherio et al., 1992; Roberts and Andrae, 2004). Increasing stocking rate (Bransby et al., 1988), use of chemical plant growth regulators (Turner et al., 1990), inter-seeding with legumes in endophyte-infected tall fescue pasture (Hoveland et al., 1981; McMurphy et al., 1990), providing a high quality forage (Tucker et al., 1989), providing protein and energy supplements (Crawford et al., 1989), supplementing blood meal as protein source (Forcherio et al., 1993) and feeding supplement like growth stimulants, anthelmintics and toxin adsorbents (Adam et al., 2006; Merrill et al., 2007) have been attempted to mitigate fescue toxicosis. Recently, supplementing vasodilators including isoflavones (Aiken et al., 2016), estradiol and soybean hulls (Aiken, 2016) has been shown to alleviate some of the toxicity symptoms.

2.6.2. Mycotoxin adsorbents

Application of conventional toxin adsorbents in feed can reduce the negative effects of endophyte toxins on animal performance (Ely et al., 2004; Wielogórska et al., 2016). Supplementation of adsorbents in the diet reduces the bioavailability of toxins in the gastrointestinal tract (Akay et al., 2003). The mean fecal ergovaline + ergovalinine concentrations of steers fed endophyte-infected tall fescue seed was significantly increased in the presence of yeast cell wall based adsorbent (FEB-200^{TM}, Alltech Inc.) compared to controls, indicating a decrease in absorption of ergovaline and its isomer in the GIT (Akay et al., 2003; Ely et al., 2004). Some have reported improved animal performance when cattle grazing E+ tall fescue were supplemented with a modified glucomannan (Ely et al., 2004; Gunter et al., 2009b). The *in vitro* evaluation of clay and yeast cell wall based adsorbents revealed dose-dependent interactions with ergotamine. However, their adsorption efficiencies were lower by nearly 51-74% in rumen fluid depending on the initial concentration of ergotamine (Dawson et al., 2001). Additionally, adsorption experiments conducted in rumen fluid have also shown decreased adsorption capacity and affinity of several adsorbents to ergotamine tartrate (Evans and Dawson, 2000). Usually, decreased adsorption in complex media occurs when the adsorbents are non-specific in binding (Whitlow, 2006; Jard et al., 2011). Therefore, evaluation of adsorbents that can achieve specific adsorption with superior affinity and selectivity is warranted. Imprinting technology is one area where many research studies have shown superior adsorption properties to target molecules.

2.6.2.1. Molecularly imprinted polymers

Biological interactions in enzyme-substrate, ligand-receptor or antigen-antibody systems are decided based on molecular recognition (Wulff and Sarhan, 1972b) and often described as lock and key mechanisms (Fischer, 1894). Researchers have used this general concept in imprinting technology to synthesize molecularly imprinted polymers (MIP) and have touted the utility of this approach in applications that require highly specific binding (Pauling, 1940). These biomimetic polymers have been shown to interact with target compounds with great specificity and selectivity (Mosbach et al., 2000; Pichon and Chapuis-Hugon, 2008). The use of MIPs has gained prominence in recent years due to low cost of production, stability in harsh conditions (pH, temperature and organic solvents) and recognition properties similar to that of natural receptors (Owens et al., 1999; Svenson and Nicholls, 2001). Additionally, by using the imprinting technique, inert macromolecular polymers with multi-functional receptor groups have been synthesized to aid in specific interactions with a targeted molecule (Haupt and Mosbach, 2000; Sellergren and Andersson, 2000; Whitcombe and Vulfson, 2001). These MIPs are synthesized using functional monomers, crosslinkers, initiator and porogen in the presence of the adsorbate of interest, called the template. The template interacts with functional monomers during polymerization, which is then networked with crosslinkers to form polymers of large molecular weight (Sellergren and Hall, 2001; Cormack and Elorza, 2004). These polymers are then washed to remove the template, thereby creating heterogeneous binding sites with functional groups (-OH, C=O, carboxyl) arranged in geometries capable of binding the template and similar molecules with varying affinities (Umpleby et al., 2004). Because of specific molecular recognition properties, MIPs have been applied in various fields, especially affinity based separation media (Haginaka, 2009), as a sorbent material for sample clean-up in analytical chemistry (Lanza and Sellergren and Hall, 2001; Martin-Esteban, 2001) and as a nanoparticle in drug delivery systems in the biomedical field (Sellergren and Allender, 2005)

2.6.2.1.1. Approaches in molecular imprinting

Essentially, three kinds of molecular imprinting strategies have been established based on interactions between the template and functional monomers 1) covalent 2) non-covalent and 3) semi-covalent imprinting.

2.6.2.1.1.1. Covalent imprinting

In the covalent imprinting approach, polymerization involves covalent bond formation between the template and monomers which possess alcohol, diol, aldehyde, ketone, primary amine or carboxylic acid functional groups (Shea and Thompson, 1978; Damen and Neckers, 1980). Upon washing, the bonds are hydrolyzed and the functional groups remaining in the binding sites have the propensity to re-establish covalent bonds with the template through a condensation reaction. This approach was pioneered by Wulff and Sarhan (1972a) who have shown very low non-specific binding due to rigid structural and functional complimentary formed during polymerization (Wulff, 1995, 2013). Other benefits of covalent imprinting are that the reversible bond between the functional groups is only associated with the template site and that functional groups responsible for binding are only located in the binding cavities, therefore restricting non-specific interactions. Additionally, it also provides the most homogeneous binding site distribution with largely identical binding pockets. Furthermore, it achieves better binding constants due to covalent bond types such as Schiff bases, boronates, ketals, carboxylic amides and esters. On the contrary, despite the high affinities of the polymeric material, they usually exhibit slow binding kinetics which restrict application in analytical applications where reversible covalent bonds are preferred. Additionally, the range of functional groups which can be targeted is limited and removal of the template molecules from the imprinted polymer is cumbersome due to the covalent linkages.

2.6.2.1.1.2. Non-Covalent imprinting

Non-covalent imprinting resembles naturally occurring recognition mechanisms and mimics host-guest biological interactions and is the most widespread method for MIP preparation, using a wide range of imprintable compounds. This approach relies on self-assembly of molecules and on interactions like ionic, dipole-dipole, π- π stacking, van der Waals, electrostatic or hydrogen bonding between monomers and the template during pre-polymerization (Arshady and Mosbach, 1981). Complexation is achieved by mixing functional monomer, template and crosslinker in a porogenic solvent matrix. It is the most widely used imprinting method owing to simpler template washing and generation of a greater number of high-affinity binding sites. This method yields a wide variety of binding sites as a result of the assembly of a complex between the monomers and template during pre-polymerization (Umpleby et al., 2004). In contrast to covalent imprinting, the self-assembly approach is characterized by a more heterogeneous binding site distribution. The most common commercially available functional monomers are

methacrylic acid, 2-hydroxyethyl-methacrylate and 4-vinyl pyridine, which have been used in a variety of applications (Kempe et al., 1993; Müller et al., 1993; Haginaka and Sanbe, 1999). 2-hydroxyethyl-methacrylate has been incorporated in methacrylate-based polymers to decrease hydrophobicity, thus allowing diffusion of the hydrophilic template (Kugimiya and Takeuchi, 1999). Additionally, with non-covalent imprinting, it is easy to fabricate the polymer by changing the backbone structure of the polymer with co-polymerization to increase the flexibility, which helps in template removal (washing) and rapid template rebinding (Mosbach, 2006b).

In non-covalent molecular imprinting, the arrangement of the functional monomers is key to affinity and selectivity, which requires the formation of stable complexes between the template and functional monomers in the pre-polymerization mixture (Sellergren and Hall, 2001). However, in the absence of complex formation, non-imprinted sites in the polymers may dilute the selective binding occurring in the templated sites. So far, many molecules have been successfully imprinted based on hydrogen bonds, electrostatic and hydrophobic interactions, albeit with some limitations encountered as a consequence of the heterogeneity of binding sites and limitations in the use of certain solvents (Umpleby et al., 2004; García-Calzón and Díaz-García, 2007). The binding affinity, which refers to the strength of binding interactions often given as a binding constant (K, mol^{-1}) or as free energy (ΔG, $kcal \cdot mol^{-1}$), is the primary parameter to evaluate the molecular recognition by non-covalent MIPs (Umpleby et al., 2004). Due to lower bond strength in non-covalent interactions, a higher molar ratio between template and monomer (1:1) is preferred to create high reuptake with superior association constants for interactions ($K_a > 10^3$ mol^{-1}) (Mayes and Mosbach, 1997; Wulff and Knorr, 2001). Therefore, optimization of polymerization conditions with proper stoichiometric ratios of the template to monomer has been suggested to increase binding properties (Lanza et al., 2002).

2.6.2.1.1.3. Semi-Covalent imprinting

The semi-covalent approach unites the advantages of the covalent and non-covalent approaches, mainly to capitalize on higher affinities provided by the covalently synthesized polymers and the faster rebinding of non-covalent polymers. During the pre-polymerization phase, the template is allowed to interact with functional groups in monomers via non-covalent interactions and the product is polymerized with crosslinkers. The polymerization is terminated by introducing oxygen (inhibitor of polymerization) or by changing the temperature and the bonds between the template and the polymers are cleaved to form complimentary

conformational and functional groups to the template used. This process allows specific interaction with the template in rebinding studies with template and its analogous compounds (Wulff, 1995, 2013). The semi-covalent approach for imprinting several compounds including 4-nitrophenol (Caro et al., 2002), triazines (Cacho et al., 2006), phenols (Qi et al., 2010) or glyceric acid (Wulff and Sarhan, 1972a) has been successful in extraction or clean-up procedures. Additionally, the semi-covalent approach has also been used in applications which required low crowding in the binding site cavity to allow unhindered non-covalent rebinding (Sellergren and Andersson, 1990; Bystroem et al., 1993). Furthermore, the use of the semi-covalent method has been adopted in nanoparticle imprinting (Poma et al., 2010), solid phase micro-extraction (Tamayo et al., 2007) and surface molecular imprinting (Curcio et al., 2009).

2.6.2.1.2. Parameters involved in molecular recognition

Parameters contributing to molecular recognition of MIPs include conformation and configuration of the polymer (Schweitz et al., 1997; Peters et al., 1999), chemical functional groups available for interactions between the polymer and template (Spivak, 2005), polarities of the binding analyte and polymer matrix (Andersson, 2000), reactivity of the binding site (ionic interactions) (Karim et al., 2005) and accessibility of the binding site (polymer porosity, density) (Henry et al., 2005). At the molecular level of MIP interaction, the template molecule should fit the internal cavity of a correspondingly designed host structure via bond fixation, coordination, and molecular recognition. A 3-dimensional cavity with multiple interaction sites will provide superior binding properties and, by increasing the number of interaction sites involved in the recognition process, the selectivity of MIP for structurally related compounds can be increased (Andersson, 2000). The non-covalent approach for MIP synthesis leads to heterogeneous binding site distribution with a small percentage of binding sites exhibiting high affinity towards the template molecule (Sellergren, 1999). Heterogeneity is the source of a high degree of cross-reactivity in non-covalently imprinted polymers and therefore it is important to understand the effects of different variables that contribute to heterogeneous binding sites during the synthesis of polymers.

At the molecular level of biological recognition, non-covalent interactions like hydrogen bonding, ion-pairing and π-π stacking contribute to the complex formation. A hydrogen bond is a strong interaction where molecules having O-H, N-H, S-H and C-H groups act as proton donors while molecules having O=P, O=S, O=C, -N= and -O- groups act as proton acceptor groups. Pi

stacking (π-π stacking) refers to the weak electrostatic attraction between molecules containing aromatic ring structures and can occur more frequently in polar solvents (water and methanol). Another non-covalent interaction is hydrophobic interaction, which is usually facilitated in highly polar solvents or solvent mixtures such as water/methanol. Several experimental variables including type of functional monomers, crosslinkers, initiator, porogen, template, temperature, pressure and thermodynamic processes contribute to interactions that consequently determine the recognition properties of the imprinted polymers (Rampey et al., 2004).

2.6.2.1.2.1. Functional monomers

Most of the research involving molecularly imprinted materials have focused on the choice of a functional monomer for the pre-polymer complex. The chemical characteristics of the functional monomers are crucial in the imprinting process and monomers are selected based on the formation of strong non-covalent interactions with the template. It is also important to match the functionality of the template with the functionality of the functional monomer in a complementary fashion. Chemical structure and properties of important functional monomers that are used in the synthesis of imprinted polymers have been well reviewed by Cormack and Elorza (2004). Normally, in the non-covalent approach of imprinting, monomers are used in excess relative to the amount of template (ratio of 4:1 and upwards) to favor the formation of template-functional monomer assemblies via increase in the functional groups. Different approaches employed for optimization and the techniques to assess interactions occurring in the pre- and post-polymerization media have been reviewed (Karim et al., 2005). The type and number of functional groups present in the template molecule have been shown to contribute to selectivity (Sellergren, 1999). Additionally, if copolymerization with more than one type of monomer is desired, the reactivity ratio of each monomer has to be considered to ensure feasibility of copolymerization (Zihui et al., 1999). Complexation of a template by a functional monomer in the pre-polymerization step has been shown to influence the reactivity of the monomer due to perturbations to the electronics and/or the sterics of the monomer (Cormack and Elorza, 2004).

Monomers are selected based on the functional groups and some of the commonly used monomers include vinyl pyridine (basic), methacrylic acid (acidic), *N,N,N*-trimethylaminoethyl methacrylate (charged), acrylamide (hydrogen bonding) and styrene (hydrophobic). These monomers usually have low association constants with the template to

form a stable complex and hence are used in excess to shift the equilibrium towards complex formation. Combinatorial approaches to MIP synthesis have been used to optimize the molar ratios of functional monomers to the template (Batra and Shea, 2003; Lanza and Sellergren, 2004) with a goal of high selectivity. Additionally, functional monomers like methacrylic acid (MAA) are usually selected for polymer synthesis in biological interactions because of their anionic acid functional groups which allow non-covalent interactions. Polymers made of MAA and acrylamide have been evaluated for binding at various pH and have been utilized for pH dependent controlled release of phytic acid (Cirillo et al., 2010b) and sustained release of 5-fluorouracil (Puoci et al., 2007b) in gastrointestinal simulating fluids. Furthermore, comparing aromatic versus non-aromatic functional monomers, the MIPs exhibiting the best selectivity were those containing aromatic amine-based functional monomers. The aromatic amines are capable of hydrogen bonding and/or electrostatic interactions with the template in a single, coplanar direction and are therefore responsible for desired MIP selectivity (Spivak et al., 1997). However, aromatic monomers are less conformationally restrained with the associated entropy gain that is known to reduce the selectivity in interactions (Simon and Spivak, 2004).

2.6.2.1.2.2. Crosslinkers

Crosslinkers have three major functions in an imprinted polymer. First, they control the morphology of the polymer matrix. Second, they stabilize the imprinted binding sites. Finally, crosslinkers provide mechanical rigidity and stability to the polymer matrix. A study conducted by Wulff (1995) showed that increasing conformational flexibility in functional monomers adversely affected selectivity due to entropically favored rotation freedom. Additionally, the decreased conformational flexibility that is usually provided by crosslinkers prevents loss of pre-organization provided by the template. High levels of crosslinkers are generally preferred to permit access to the interior of the porous cavity in the polymer matrix. Approximately 80-90% of imprinted polymers are comprised of the cross-linking monomer that holds the functional monomers in a certain orientation. As discussed earlier, the reactivity ratio of the crosslinkers also plays an important role in the imprinting and polymerization process (Gelfi and Righetti, 1981; Yoshimatsu et al., 2007). Quite a number of crosslinkers compatible with molecular imprinting have been in use, many of which are commercially available, including ethylene glycol dimethacrylate (EGDMA), tetramethylene dimethacrylate (TDMA), N, N-methylene bisacrylamide (MDAA) and divinylbenzene (DVB).

2.6.2.1.2.3. Initiator

Free radical polymerization is the most common synthetic method available today for the conversion of vinyl monomers to polymers under mild reaction conditions (e.g., ambient temperatures and atmospheric pressures). Free radical formation is usually initiated by chemical, thermal or photochemical methods. Many of the chemical initiators are either peroxides or compounds containing azo groups. Some of the commonly used initiators in imprinting technology are azobisisobutyronitrile, dimethyl acetal of benzyl, benzoyl peroxide, azobisdimethylvaleronitrile and 4,4'-azo(4-cyanovaleric acid). These are normally added at very low concentrations compared to the monomer (1 wt. %, or 1 mol. % with respect to the total number of moles of polymerizable double bonds). Typically, among the three stages of polymerization using free radical polymerization (initiation, propagation and termination) the rate of propagation is much faster than the rate of initiation (Billmeyer, 1971) and the free radicals are active over the entire period of polymerization (Xia and Matyjaszewski, 1997).

During polymer synthesis, care must be taken to reduce oxygen gas that is known to retard free radical polymerizations that can affect propagation and to disturb batch-to-batch reproducibility of the polymer. Oxygen gas can be removed either by ultrasonication or by sparging of the monomer solution by an inert gas, e.g. nitrogen or argon. Additionally, where complexation is driven by hydrogen bonding, lower polymerization temperatures and photochemically active initiators are preferred. An azo-initiator (azobisisobutyronitrile; AIBN) is easily decomposed by photolysis (UV) or thermolysis to provide carbon-centered radicals capable of initiating polymerization of vinyl monomers (Yan and Row, 2006). Photo-initiation polymerization is conducted at low temperatures compared to thermally initiated polymerization (>40 °C) and has been shown to increase the binding capacity and specificity due to low kinetic energy of the pre-polymerization complex, providing stability to the polymer matrix (Spivak et al., 1997; Puoci et al., 2007a; Athikomrattanakul et al., 2009).

2.6.2.1.2.4. Porogen

Porogen solvent brings all the components of polymerization into one phase and plays a critical role in the formation of the porous structure and interactions between functional monomers and template in the pre-polymerization step of MIP synthesis (Song et al., 2009). In non-covalent interactions, a wide variety of possible bond types implies that molecular recognition of a guest molecule may be dominated by a combination of different recognition

mechanisms, which are enabled or disabled depending on the polarity of the selected protic or aprotic porogen (Theodoridis et al., 2004). Non-polar solvents like chloroform and toluene are commonly used when it is critical for the solvents not to interfere with the hydrogen bonding/ionic interactions between monomers and template molecules. Additionally, porogenic solvents with low solubility phase separate early and have been shown to produce large pores and low surface area, while porogens with higher solubility phase separate later in polymerization and produce small pore distribution with greater surface area (Yan and Row, 2006). An ideal porogen should produce large pores that provide good flow-through properties of the resulting polymer and also should have relatively low polarity to reduce interference during complex formation between the imprint molecule and the monomer. Additionally, all the components of polymerization should be compatible in the porogen solvent.

2.6.2.1.2.5. Template

The template is the key molecule that directs the organization of functional groups in the molecular imprinting processes. Templates are selected based on their chemical and physical properties including the shape, size, functional groups and solubility in organic solvent. Most importantly, the template should be chemically inert and stable under the polymerization conditions, especially in free radical polymerization induced by the thermo-chemical or photochemical method. Some templates have been shown to inhibit or retard free radical polymerization, especially templates posessing a thiol group or a hydroquinone moiety (Cormack and Elorza, 2004; Pichon and Chapuis-Hugon, 2008). Additionally, when the goal is to bind structural analogs, the template should be chosen based on the overall representation of the target analytes. Any variation from the structure of the desired species to a structurally similar entity may result in loss of selectivity. Furthermore, when cost and procurement have presented challenges, dummy templates have been used that were close in structural conformation and functional groups (Theodoridis et al., 2003; Pichon and Chapuis-Hugon, 2008). In one study, an 82% recovery was observed when ergometrine was used as a dummy template to synthesize methacrylic acid (MAA) based MIP to extract lysergic acid diethylamine (LSD) from hair and urine samples (Chapuis-Hugon et al., 2009). Similarly, MIP with MAA monomer, cross-linked with EGDMA in chloroform, that was imprinted with metergoline template exhibited high selectivity towards LSD when compared to non-imprinted polymers (NIP) (Lenain et al., 2012). However, cross-reactivity with certain polycyclic compounds was noticed with both MIP and NIP.

The use of toxic compounds (like ergot alkaloids) in the synthesis of imprinted sites in polymer molecules may cause a toxicity risk to the researcher, users, and the environment. In such situations, it is advantageous to use a nontoxic chemical as a structural analogue in place of the toxic compound to enhance laboratory and environmental safety. Another disadvantage of using pure compounds is the release of template from the imprinted sites in a process known as template bleeding. Toxic compounds intended as templates can be replaced by mimotopes. Mimotopes are compounds which have 3-dimensional conformation and geometries similar to compounds of interest. Researchers have synthesized mimotopes for various mycotoxins e.g., OTA, ZEN, DON (Lai et al., 2004; Bazin et al., 2013; He et al., 2013) and have used these for rapid and accurate detection of mycotoxins.

2.6.2.1.2.6. Temperature, pressure and thermodynamic processes

An important consideration for the success of the imprinting process is the maintenance of the monomer-template complex during the pre-polymerization step (Shea et al., 2002). Generally, the affinity and specificity of polymers could be significantly improved by decreasing the polymerization temperature (Piletsky et al., 2002; Piletsky et al., 2004; Piletsky et al., 2005). A high temperature is expected to drive the equilibrium away from the template-functional monomer complex toward the unassociated species, resulting in a decrease in the number of imprinted cavities. The stability of complexes is under thermodynamic control and binding energetics (ΔG_{bind}) involved in complex formation can be attributed to the sum of translational and rotational (ΔG_{t+r}), rotor restriction upon complex formation (ΔG_r), hydrophobic interaction (ΔG_h), residual soft vibrational mode (ΔG_{vib}), sum of interaction polar group contributions ($\sum \Delta G_p$), adverse conformation changes (ΔG_{conf}) and unfavorable van der Waals interaction (ΔG_{vdW}) (Holroyd et al., 1993). Lower temperatures have been shown to favor complexation due to a reduction in the influence of residual vibrational modes (ΔG_{vib}) and an increase in the strength of polar interactions ($\sum \Delta G_p$) (Sellergren, 1989; Holroyd et al., 1993). Comparing polymers prepared using different initiators at temperatures between 0 and 60 °C, showed that MIPs synthesized at low temperature have better specificity than those prepared at a higher temperature (O'Shannessy et al., 1989; Lu et al., 2004). Studies also showed that polymers synthesized at low temperature produced homogeneous particles but had lower surface areas and pore volumes (Sellergren and Shea, 1993; Piletska et al., 2009). Additionally, the polymerization reaction is exothermic in nature and increasing the volume of the monomer

mixture resulted in pronounced increases in the temperature within the reactor that adversely affected the adsorption properties of the polymer (Piletska et al., 2009).

Additionally, glass transition temperature is one of the important factors governing the stability of amorphous materials. Temperature and time dependent changes in the physical state can affect the adsorption properties of polymers. Moisture acts as a plasticizer and significantly changes the glass transition temperature of amorphous solids (Roos and Karel, 1990; Sablani et al., 2007) leading to increased free volume and a weakening of the interchain interactions (Roos and Karel, 1990). Polymers may be stable below glass transition temperature because deterioration due to chemical reactions is greatly reduced. Additionally, molecular diffusion is extremely slow if the polymer is in a glassy state in aqueous media that could affect the adsorption profile irrespective of the polymer type.

Some studies have attempted to analyze the role of pressure on the performance of MIP. Sellergren et al. (1997) showed that MIPs synthesized under high pressure compared to ambient pressure had higher affinities due to increased intramolecular associations in the monomer mixture. Another study also showed substantially higher affinity of polymer prepared at 10 bars compared to polymer prepared at ambient pressure (Piletsky et al., 2005). Additionally, increasing the pressure by 1000 bars increased the propagation rate of polymerization by a factor of 1.1 in acrylate and methacrylate-based polymeric systems (Beuermann and Buback, 2002). Furthermore, studies have also suggested that the boiling point of the solvent affects the rate of polymerization (Beuermann and Buback, 2002; Piletsky et al., 2005).

2.6.2.1.2.7. Template bleeding

In some imprinted polymers prepared using the non-covalent approach, it has been shown that the binding of the template to the polymer is so strong that it is difficult to remove the last traces of template, even after washing the polymer several times (Andersson et al., 1997; Martin et al., 2003). Chemically, the non-covalent interactions are attenuated using mild acidic or basic organic solvents which facilitate removal of the template from the polymerized network. However, sometimes it is very difficult to remove the template from MIP completely, leading to a phenomenon called “template bleeding” (slow release of the remnant template into the media). Template bleeding has been related to leakage of the physically entrapped template during swelling or shrinkage of the polymer material, or to the formation of template

clusters that could be released by the polymeric material (Sellergren and Hall, 2001). Template bleeding is one of the recognized drawbacks of MIP application as solid phase extraction material (Ellwanger et al., 2001). Even though harsh washing techniques like thermal annealing, microwave assisted extraction, Soxhlet extraction and supercritical fluid template desorption (Ellwanger et al., 2001; Peng-Ju et al., 2007) have been attempted, the success rate in complete recovery of the template from the imprinted polymers is low. To overcome this drawback, some researchers have synthesized MIP using an analog (dummy template) of the target molecule as a template (Andersson et al., 1997; Dirion et al., 2002).

2.6.2.1.3. Polymer synthesis

Polymer synthesis methods to prepare MIPs have been well reviewed (Yan and Row, 2006). Some of the important available polymerization methods include bulk, dispersion/precipitation, multi–step swelling, suspension, surface and in-situ polymerization.

2.6.2.1.3.1. Bulk polymerization

Most molecular imprints have been synthesized by bulk polymerization where the functional monomers have been prearranged around a template molecule in an organic solvent and copolymerized with an excess of crosslinkers in the presence of free radical initiator induced by either thermal or photochemical methods. The resulting block polymer is then crushed to the desired particle size and the template is washed. The polymers thus prepared are irregular in shape with a wide range in particle size distribution and have been often utilized as solid phase extraction material due to good specificity in binding (Al-Kindy et al., 2000; Holthoff and Bright, 2007; Petcu et al., 2009). However, some studies have identified disadvantages of bulk polymerization, including a complicated synthesis process, low recognition binding sites within the polymer matrix and poor binding kinetics for the template molecules (Pichon and Chapuis-Hugon, 2008; Beltran et al., 2010).

2.6.2.1.3.2. Precipitation polymerization

Precipitation polymerization is preferred over bulk polymerization to synthesize regularly shaped and monodispersed particles of polymers that have better binding properties. Basically, the synthetic process starts with very dilute monomers that are allowed to propagate in solution to reach a critical mass and then precipitated from the solution by decreasing the solubility using a large amount of crosslinkers. The polymer beads are separated by centrifugation and then washed. This technique offers an efficient methodology for the

synthesis of MIP beads and has been applied in the preparation of MIP nanospheres for capillary electrochromatography (Nilsson et al., 2004; Turiel and Martin-Esteban, 2005; Priego-Capote et al., 2008). However, the limitation of this approach is that the monomers should be soluble in the solvent while the resulting beads should be insoluble and the beads may aggregate resulting in inferior mass transfer properties. To overcome the problems of aggregation, some have used multi-step swelling and polymerization that also produced spherical particles (Beltran et al., 2010; Gokmen and Du Prez, 2012).

2.6.2.1.3.3. Multi-step swelling and polymerization

In this approach, uniformly sized seed particles are suspended in water and allowed to swell to a certain pre-determined range (usually 5-10 μm) by several additions of appropriate organic solvents. The components involved in the production of MIP are then added to the solution during the swelling stage and polymerization is initiated (Beltran et al., 2010). A uniformly sized molecularly imprinted polymer prepared by multi-step swelling and polymerization using methacrylic acid for d-chlorpheniramine showed superior selectivity towards template compared to non-imprinted polymer and slight recognition for its structurally related compounds (Haginaka and Kagawa, 2002). In another study, methacrylic acid-based MIP non-covalently prepared by a two-step swelling technique using isomers of diaminonaphthalene or a chiral amide derived from (S)-α-methylbenzylamine as the template molecule have been shown to enhance chiral recognition (Hosoya et al., 1996). However, even though polymer particles are relatively monodispersed in size and shape and appropriate for chromatographic applications, fairly complicated procedures and reaction conditions are required. Additionally, the requirement for aqueous suspensions (emulsion) used in this technique could interfere with the imprinting leading to a decrease in selectivity.

2.6.2.1.3.4. Suspension polymerization

A different approach to preparing spherical particles is to use suspension polymerization. In this method, all the components are dissolved in organic solvent and later transferred to a larger volume of immiscible solvent. The mixture is stirred vigorously to facilitate droplet formation and then subjected to polymerization induction. Imprinted polymers synthesized using acrylamide monomers for bovine serum albumin using this technique exhibited good recognition for template proteins as compared to the control protein and also showed better adsorption kinetics of MIP (Pang et al., 2005). Using this two-phase system,

regular MIP microspheres have been synthesized that exhibited an excellent chromatographic performance with superior selectivity, even at high flow rates (Mayes and Mosbach, 1996; Zhang et al., 2003). Unfortunately, this approach uses immiscible liquids (e.g., perfluorocarbons) instead of water as the continuous phase to avoid the detrimental effect of water on the non-covalent complex between monomers and imprint molecule thereby imposing limits on the applicability and practicality of this method.

2.6.2.1.3.5. Surface imprinting

Surface imprinting is another polymerization technique aimed at delivering spherical particles with an imprinted layer grafted on the surface (Pichon and Chapuis-Hugon, 2008; Beltran et al., 2010). This approach is well suited to make the coating on chromatography-grade porous silica or spherical polymers (Vasapollo et al., 2011). Here, all the components involved in the polymerization process are absorbed into the beads or silica particles and then polymerized. The silica particles or the beads are then etched away to reveal the final product that retains the shape of the original bead or silica particle (Komiyama et al., 2003; Tan and Tong, 2007). This surface imprinting technique was used to prepare metal-ion imprinted membranes by water-in-oil emulsion polymerization (Araki et al., 2005). Superior chiral selectivity towards the original template was observed with surface-initiated radical polymerization of ultra-thin MIP films grafted on gold-coated quartz crystal (Piacham et al., 2005). Despite their good recognition properties in aqueous media, these membranes were not stable in an organic environment.

2.6.2.1.3.6. In-situ polymerization

In-situ polymerization uses interpenetration of polymer networks by crosslinking and are commonly used to synthesize MIP membranes. The in-situ polymerization technique was first used for the preparation of molecularly imprinted monoliths (Matsui et al., 1993). Template, initiator, monomer and crosslinker are dissolved in porogenic solvents and poured into a stainless steel column and polymerized. The template and porogenic solvent are removed by thorough washing with acidic methanol solvent to form a monolithic molecularly imprinted polymer. This technique has advantages including ease of preparation, high reproducibility, high selectivity and sensitivity, and rapid mass transport. Their great porosity, and hence good permeability, and high surface area are well suited for both small molecules and large biopolymers. In recent years, monolithic molecularly imprinted stationary phases have become popular in chromatographic stationary phase preparations (Liu et al., 2005). However, some

conflicting results were also reported showing low membrane permeability (Sergeyeva et al., 2007; Vasapollo et al., 2011) which was suggested to be an obstacle for application in separation technology.

2.6.2.1.4. Polymer characterization

The quality of MIP is usually assessed by chemical properties, morphological properties and binding characteristics.

2.6.2.1.4.1. Chemical characterization

Elemental microanalysis measures the percentage by mass of elements within samples that can be used to calculate the co-monomer composition of the polymer. Co-monomer compositions are easily calculated when one of the co-monomers bears a heteroatom, e.g. nitrogen in the 4-vinylpyridine co-monomer in poly (4-vinylpyridine-co-divinylbenzene), but this approach is not sensitive enough to detect residual traces of template in the imprinted polymer. Another method to obtain quantitative information on the composition of the polymer is the use of spectral information from infrared spectroscopy. Infrared spectroscopy provides information about the structure and local environment of polymer chains that is of diagnostic value when diverse chemical environments arise from the functional monomer and crosslinker in an imprinted polymer (Bellamy, 2013). Additionally, Fourier-transform infrared spectroscopy (FTIR) is used to probe available functional groups that may contribute to interactions between the template and the polymer (Karim et al., 2005). Furthermore, ^{1}H-NMR and ^{13}C-NMR are useful tools for the determination of regularity and tacticity in soluble polymers (Cowie and Arrighi, 2007). However, for insoluble polymers, solid state ^{13}C-NMR (SS-NMR) has so far provided the most useful information (Hjertberg et al., 1990; Law et al., 1996; Díez-Peña et al., 2002; Qian et al., 2010) that estimates unreacted double bonds in the polymer and the relative amount of different types of carbon atoms based on cross-polarization magic angle spinning.

2.6.2.1.4.2. Morphological characterization

Variation in the morphological and chemical properties as a result of the imprinting process can affect the dynamic binding properties of the synthetic receptor sites. Mass-transfer kinetics are also related to the physical characteristics of the amorphous polymers and can be probed using the same techniques that are used for any most porous solids. A study conducted by Sellergren and Hall (2001) demonstrated that the diffusion coefficient of the analyte was dependent on its affinity to binding pockets, the dimensions of the void spaces in the cross-

linked polymer, and the size of the permeant. Increasing permeant volume has been shown to change the dimensions of polymers (swelling effect) by imposing internal stress on polymer chains, thus affecting the integrity of binding pockets. A relatively high degree of crosslinkers can be used to maintain the structural integrity of cavities in the imprinted polymer (Cormack and Elorza, 2004). However, the association and dissociation rates of host-guest complexes formed within the embedded cavity were compromised due to loss of flexibility in polymers. Therefore, the structure and morphology of the polymers are generally evaluated to determine the accessibility and microenvironment of the functional groups and binding sites to aid in understanding the permeation/diffusion and binding properties of the polymers.

2.6.2.1.4.2.1. Gas sorption measurements

For gas sorption, a gas (usually nitrogen) is dosed in small amounts into a degassed sample chamber containing a known amount of dry polymer at the gas boiling point. By measuring the amount of gas absorbed as a function of pressure at equilibrium, morphological details (specific surface area (m^2/g), specific pore volume (mL/g), average pore diameter, and pore size distribution) are obtained from adsorption isotherms described by S. Brunauer, B. H. Emmett, and E. Teller (Brunauer et al., 1938; Barrett et al., 1951). Six types of isotherms have been used to gain information on porosity (Sing, 1982). The method is particularly useful for analyzing the particle size in the nanometer range (micropores <2 nm; 2 nm < mesopores <50 nm; 50 nm< macropores).

Polymers made using MAA/EGDMA revealed type IV isotherms indicating mesoporous structure with surface areas ranging between 100 and 400 $m^2 \cdot g^{-1}$ (Sellergren and Shea, 1993). The same study showed that polymers made with chloroform as porogen were non-porous and had a low surface area (3.5 $m^2 \cdot g^{-1}$). The main factors that have influenced the physical properties of imprinted polymers have been type and amount of porogen, the percentage of crosslinkers and polymerization conditions (Spivak, 2005; Wei and Mizaikoff, 2007; Holland et al., 2010). Comparing different porogens, polymers made using chloroform or toluene (hydrophobic solvent) had lower surface area than polymers made in acetonitrile (Kyzas et al., 2009). That study also demonstrated that increasing the amount of cross–linkers (EGDMA) decreased the average pore diameter and number of binding sites of MIP (Holland et al., 2010).

2.6.2.1.4.2.2. Particle size distribution

The particle size distribution of a polymer sample is measured using laser diffraction built upon the “Mie theory” of the physical principle of the interaction between the light beam and the particle (Kyzas et al., 2009). The range of particle size distribution as a function of monomer and polymer conversion, chain length propagation and rate of chain length termination during polymerization is described by the power law (Matyjaszewski and Davis, 2002). Even though the polymers are ground and sieved to a particular particle size in the case of bulk polymerization, studies have shown that solvent used in rebinding studies can affect the morphology of the polymer (swelling effect) and the binding properties (Farrington and Regan, 2007; Yoshimatsu et al., 2007) suggesting the need for evaluation of the particle size distribution in the solvent that is used in rebinding studies. These same studies indicated that water caused the least reduction in average particle size of MIP compared to methanol and acetonitrile.

2.6.2.1.4.2.3. Electron Microscopy

Scanning electron microscopy (SEM) and tandem electron microscopy (TEM) techniques are the best tools to examine the surface topography (surface features of the sample), morphology (shape and size) with excellent resolution (10nm) (Thomas and Gai, 2004). In electron microscopy, the electron beam is scanned across the surface of the sample, with detectors building up an image by mapping the detected signals with beam position (Sundar et al., 2010). Samples are coated with a heavy metal nano-film in order to reduced sample charging by increasing electrical conductivity. Studies have shown that morphology and structural properties of MIPs are important factors that affect the binding behavior of the polymers which can be controlled by manipulating polymerization conditions (González et al., 2006).

2.6.2.1.4.3. Binding characterization

The adsorption properties of the MIP are usually assessed either with affinity chromatography or isothermal adsorption studies. Affinity chromatography (AC) is a type of liquid chromatography that uses a ligand as the stationary phase (Hage, 2002). In this method, one of a pair of interacting compounds is immobilized onto a support for use as a stationary phase, while the other compound is contained within the injected sample or mobile phase. These support materials are often used when purifying samples under gravity flow or using a peristaltic pump. High-performance affinity chromatography (HPAC) is a modification on this method in which the support material is made of small, rigid particles that are able to withstand

high back-pressures. Typical supports in HPAC include silica and polymeric monolith materials. The binding properties are evaluated based on the separation or elution profile from the support materials in different solvents, which can provide information regarding affinity and capacity. Chromatographic separation has been useful in identifying enantioselective MIPs that have receptor sites capable of discriminating optical isomers (Wulff and Vesper, 1978; Sellergren and Shea, 1993; Sellergren and Hall, 2001) in the 3D binding cavities that are complementary in size, shape, and functionality to the template (Kempe and Mosbach, 1995). Chromatographic studies have also been used to determine the influence of chemical properties of the template, hydrophobicity and selective molecular shape recognition in organic and aqueous media (Vlatakis et al., 1993; Yu and Mosbach, 1998).

Alternatively, MIPs are frequently evaluated using isothermal adsorption experiments (rebinding experiments) and studies have demonstrated comparable recognition properties to that of ligand-receptor interactions in biological systems (Vlatakis et al., 1993; Ye et al., 1999). After the template has been extracted, the MIP is immersed in solutions of varying concentration of the template. The change in concentration of the solution as the template is reabsorbed is measured, typically using HPLC-mass spectrometry or UV spectroscopy (Vlatakis et al., 1993b; Haupt, 2003). The advantage of using binding isotherms is the ability to compare the adsorption parameters from different studies conducted at different times (Atwood and Steed, 2009). However, due to heterogeneity in interactions with MIPs, evaluating the binding properties of MIPs sometimes becomes complicated (Andersson and Nicholls, 1997).

Accurate analyses of the mechanisms of molecular recognition in MIPs are central for the development of synthetic receptors using rationally controlled parameters during synthesis of polymers. Due to the amorphous nature of imprinted polymers, structural modeling of binding sites in the imprinted polymers cannot be determined by crystallographic or microscopic methods. Theoretically, the number of binding sites is proportional to the amount of template used in pre-polymerization of MIP synthesis because selective binding sites would be formed at the point of contact. Binding parameters, including affinity and capacity, are usually estimated from mathematical adsorption isotherm models. One of the important analytical requirements is for the data to be collected after the equilibrium between the template and the MIP has been reached. Generally, the free template concentration after incubation with a known amount of polymer is measured after a specified period of time necessary to reach equilibrium (Sellergren

and Hall, 2001). Some of the isotherm models used include Scatchard, Langmuir and its derivative models, Freundlich and its derivative models and combinations of Langmuir and Freundlich models.

2.6.2.1.4.3.1. Scatchard analysis

One strategy to investigate the binding performance of polymers is based on Scatchard plot analysis on data obtained from saturation studies. Scatchard plots yield a slope –K (association constant) and intercept (B_{max}) by plotting the ratio of bound to free template concentrations versus the bound concentrations, under the assumption that all binding sites are identical and independent (Table 2-2). However, with the presence of heterogeneous binding, the Scatchard plot usually results in a curve with the degree of curvature containing information on the heterogeneity of the binding sites. With the limiting assumptions made i.e., a bimodal distribution of binding sites and neglecting non-specific binding within the selected concentration range, Scatchard analysis estimates dissociation constant and the number of binding sites (Scatchard, 1949; Voet and Voet, 2004). However, with non-covalent approach, more heterogeneous binding sites are created and the approximation of adsorption parameters limited to bimodal distribution by Scatchard plot may not account for full description of the binding properties. Therefore, models including Langmuir for homogeneous binding sites (Langmuir, 1918) and Freundlich for heterogeneous binding sites (Freundlich and Hatfield, 1926) have been frequently used to describe the affinity and maximum binding parameters.

2.6.2.1.4.3.2. Langmuir isotherm

Langmuir isotherms quantitatively describe a homogenous binding system with monolayer adsorption between solids and liquids and assume identical adsorption sites of uniform energies (Langmuir, 1918; Whitcombe et al., 1995). The model holds good when there is a finite number of adsorption sites on the outer surface of adsorbent and no transmigration of adsorbate on the plane of the surface (Langmuir, 1918; Vermeulan et al., 1966; Belton, 1976). The important features of Langmuir isotherms are the adsorption constants K_L and R_L (Table 2-2), that refer to the energy of adsorption (Weber and Chakravorti, 1974) and favorability of adsorption (Nwabanne and Igbokwe, 2008), respectively. The energy of adsorption depends on the activity coefficient of occupied and unoccupied sites on the adsorbents at equilibrium (Graham, 1953) that relates to the molar concentrations of adsorbate in the media. A positive K_L indicates that the interactions are spontaneous and energetically favorable (Sun et al., 2013).

The R_L value is called the separation factor, a dimensionless constant which predicts the affinity of the adsorbate to the adsorbent and a value between 0 and 1 signifies favorable adsorption (Foo and Hameed, 2010; Sun et al., 2013). Even though Langmuir isotherm provides informative adsorption parameters, it does not offer an accurate description of the binding behavior of non-covalent interactions in MIPs, which has led to the utilization of Freundlich isotherms that take into account the heterogeneity in binding sites.

2.6.2.1.4.3.3. Freundlich isotherm

With heterogeneous binding, a wide range of interactions is accurately modeled by an exponential decay binding model known as a Freundlich isotherm, which describes a particular number of binding sites having a particular association constant (Freundlich and Hatfield, 1926; Umpleby et al., 2001a; Umpleby et al., 2004). Freundlich isotherms describe heterogeneous surface adsorption on the adsorbent with multiple layers of binding (Hutson and Yang, 1997). The Freundlich constants, K_f and 1/n, are the parameter characteristics of the sorbent- sorbate system (Table 2-2). The constant K_f is a function of the energy of adsorption which indicates adsorption capacity, whereas n measures heterogeneity in adsorption (Voudrias et al., 2002). The Freundlich constant (n) is also used to predict favorability of adsorption where high n values indicate strong interaction with greater heterogeneity in adsorption. Generally, heterogenic interactions are common between organic molecules in the presence of electronegative groups (Takahashi et al., 2010), Van Der Waals interactions (Thallapally and Nangia, 2001) and hydrophobic interactions (Zhongbo and Hu, 2008). Some of the examples are methylene blue adsorption onto palm kernel fiber in aqueous solution (Ofomaja, 2007), tartrazine onto hen feather (Mittal et al., 2007) and toxic molecules onto activated charcoal (Gessner and Hasan, 1987). Occasionally, adsorbents with heterogeneous binding sites are known to fit both Langmuir and Freundlich models (Umpleby et al., 2001b; Okeola and Odebunmi, 2010), but most frequently, a better fit with Freundlich has been observed, indicating the presence of heterogeneous binding sites with different binding affinities (Okeola and Odebunmi, 2010), and selectivities (Umpleby et al., 2001b). Free radical polymerization with concentrated solutions of monomers and high levels of crosslinkers typically yield heterogeneous polymers. Such polymers have been shown to possess numerous non-uniform distributions of binding sites producing non-linear adsorption plots (Sellergren and Shea, 1993; Umpleby et al., 2001b).

2.6.2.1.5. Application of imprinting technology in analytical chemistry

Due to the ease of synthesis, stability in harsh conditions (temperature, pressure, organic solvents and chemicals) and ability to mimic biological receptors in binding selectivity and sensitivity, MIPs have been useful in a wide range of applications in analytical chemistry.

2.6.2.1.5.1. Chromatography and Capillary Electrophoresis

Much of the early work in chromatographic separation was done using a non-imprinted column stationary phase. The use of imprinting technology in chromatography was pioneered by Wulff in the 1970s (Wulff, 1982). Much of the later work using imprinting technology was devoted to utilizing MIPs synthesized from non-chiral monomers as stationary phases in affinity chromatography for the enantio-separation of racemic mixtures of chiral compounds (Sellergren et al., 1988; Haupt and Mosbach, 1998; Dzgoev and Haupt, 1999; Haupt et al., 1999). One key aspect of MIPs is that they can differentiate enantiomers of compounds containing more than one stereogenic center (Ansell, 2005). A significant stereoselective recognition was noticed for the resolution of cinchona alkaloids in chromatographic separation using an amino acid derivative as a chiral functional monomer and hydroquinidine as the pseudo-template molecule (Zhou et al., 2012). Several research studies have been conducted on chiral compound separation utilizing MIPs, especially using amino acids and their derivatives as templates (Nicholls et al., 1995; Dauwe and Sellergren, 1996; Mayes and Mosbach, 1996). Other classes of compounds that have been used in chromatographic studies include pesticides (Tamayo and Martin-Esteban, 2005), peptides (Nicholls et al., 1995), sterols (Hwang and Lee, 2002a), and sugar derivatives (Wulff, 1995). Additionally, evaluation of MIP in electrical driven separations using MAA-EGDMA polymers imprinted with L-phenylanaline analide, benzamide or pentamide was carried out directly in a capillary electrophoretic system (Nilsson et al., 1994; Lin et al., 1996). Additionally, other classes of compounds that have been separated using capillary electrophoresis include propranolol (Schweitz et al., 2002), ephedrine (de Boer et al., 2002), fungicides (Cacho et al., 2008) and amino acids (Lin et al., 1996). Even though benefits of the use of MIP in chromatography have been identified, limitations in its utility have been acknowledged due to the heterogeneity of binding sites and restricted permeability. Additionally, due to the difference in the binding strength of various imprinted sites, tailing in chromatographic peaks has been noticed (Sellergren and Shea, 1993).

2.6.2.1.5.2. Sensors

Surface imprinting that forms recognition sites at the surface membrane has been utilized in chemical sensing device as an alternative to immobilized bio-receptors such as antibodies and enzymes. During the past decade, studies on the development of MIP-based sensors, including electrochemical (Kan et al., 2008; Riskin et al., 2008) and optical (Li et al., 2007; Feng et al., 2008) sensors have increased dramatically. However, slow diffusion rate, slow rebinding kinetics and limited reproducibility of imprinted membrane properties are limiting parameters and may render the application of MIPs in chemical sensor technology unfeasible. Furthermore, for applications in the fields of clinical diagnostics and environmental monitoring, these sensors require selective recognition in aqueous conditions that remains a challenge for most MIP receptors.

2.6.2.1.5.3. Extraction technology: Solid phase extraction

Imprinted polymers are efficiently utilized as solid phase extraction materials (MISPE) to reduce cumbersome sample preparation steps and facilitate selective isolation, sample purification, and pre-concentration of chemical species (Sellergren, 1994; Andersson et al., 1997; Muldoon and Stanker, 1997; Zander et al., 1998). Application of MIPs, especially as MISPE extraction material, have been well researched for compounds including mycotoxins (Pascale et al., 2008), sterols (Puoci et al., 2008a), various drugs (Beltran et al., 2009), amide anesthetics (Andersson et al., 2002) and organic dyes (Li et al., 2008). The majority of the MISPE studies indicated that MIPs are stable under strong elution conditions including a wide range of pH, different solvents, and various organic modifiers (Lai et al., 2004; Say et al., 2004; Caro et al., 2006; Luo et al., 2008). Additionally, one study has confirmed that MIPs are more selective than C_{18} or ion exchange materials that are traditionally used in sample treatment methods like solid phase extraction, and are also more selective and stable than immuno- extraction matrices (Pichon et al., 1999). The utility of MISPE in extraction of target analytes from biological samples including blood plasma/serum (Wilson et al., 1997), urine (Berggren et al., 2000), bile (Wilson et al., 1997), liver extracts (Muldoon and Stanker, 1997) have also been well established. A considerable improvement in the accuracy and precision of HPLC methods, along with a four-fold decrease in the detection limit and increased recovery from 61 to 89% was shown using MISPE, as compared with non-imprinted polymer, as the sample clean-up material for the analysis of the herbicide atrazine in beef liver samples (Muldoon and Stanker, 1997). The

improved detection limit in that study was attributed to the removal of interfering compounds resulting in greater resolution of the atrazine peaks.

Additionally, with high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS), clean up of samples prior to analysis is very important to avoid matrix interference due to ion suppression or enhancement (Sellergren, 1997; Alexander et al., 2006; Yan and Row, 2006). The MISPE approach can serve as an alternative to liquid-liquid extraction, centrifugation and filtration. Studies have shown that MISPE developed for ergot alkaloids using metergoline as a template and acrylates as functional monomers possess specificity and recovery similar to ion-exchange or immune-affinity columns (Lenain et al., 2012). Furthermore, polymers targeting lysergic acid diethylamide (LSD) that were developed using ergometrine as the template molecule also showed superior binding to the template (Chapuis-Hugon et al., 2009) indicating its utility as sorbent material in MISPE columns. However, one of the drawbacks in MISPE is the bleeding of template leading to potentially large errors in the precision of the analytical measurement, which can be circumvented by imprinting with dummy template (Kriz et al., 1994; Andersson et al., 1997; Quaglia et al., 2001).

Extraction and sample clean-up prior to detection and quantification is of considerable importance in ergot alkaloid analysis because those alkaloids are encountered in a variety of biological samples and background matrices. Developments in instrumental techniques have given us the ability to separate and measure individual ergot compounds and their isomers, and thereby has allowed the possibility of monitoring and regulating the contamination of feed and food. In order to better study the effects of alkaloids, and their toxicokinetics, precise analytical tools that measure the alkaloids in parts per billion concentrations in various matrices are necessary

2.7. Ergot alkaloid analysis

Ergot alkaloid analysis has evolved substantially, from the earliest analysis of contamination based on physical counts of sclerotia in grain samples to estimates based on near-infrared hyperspectral image analyzing fat and starch content in ergot bodies, which differ from those of normal cereals (Vermeulen et al., 2013) to sophisticated method like LC-MS/MS (Di Mavungu et al., 2012). Different methodologies for the determination of ergot alkaloids in grains, grasses, feeds and grain foods including colorimetric, chromatographic and enzyme-linked

immunosorbent assay (Shelby and Kelley, 1992; Schnitzius et al., 2001), along with instrumental procedures such as liquid chromatography (HPLC) with fluorescence (Yates and Powell, 1988; Rottinghaus et al., 1991), mass spectrometric (MS) or MS/MS detection (Yates et al., 1985; Smith et al., 2009), capillary zone electrophoresis (Frach and Blaschke, 1998) and direct MS/MS have been reviewed (Scott, 2007).

An enzyme-linked immunosorbent assay (ELISA) method was developed recently and has been used to determine the total ergot alkaloid concentrations (Tunali et al., 2000; Ayers et al., 2009). However, the ELISA method has been criticized for lack of specificity (Agee and Hill, 1994), cross-reactivity (Cody and Valtier, 1997), inability to differentiate epimers (Hopkins et al., 2010), false positive results and inconsistent results (Cody and Valtier, 1997; Schnitzius et al., 2001). Studies have shown low binding efficiencies to the monoclonal antibodies; only 50% binding efficiencies for lysergic acid (at 10^{-7}mol/L) and ergonovine/ergonovine maleate (at 10^{-8} mol/L; Schnitzius et al., 2001). The driving forces for the conversion of alkaloids between their epimeric forms are complex and not fully understood (Andrae et al., 2014). Several factors that influence epimerization including the type of alkaloid, extraction solvent, temperature, irradiation, pH and storage time (Hafner et al., 2008; Andrae et al., 2014) have been shown to cause analytical bias. Although ELISA permits a quick determination of total ergot alkaloids using commercially available kits (Hill and Agee, 1994), the HPLC-fluorescence method has the advantage of distinguishing individual ergot alkaloids (Müller et al., 2006; Storm et al., 2008).

In an early study, *Neotyphodium coenophialum* culture media was extracted with chloroform and tartaric acid for ergot alkaloid analysis by chromatography (Bacon, 1988), which was then refined for complex media, with chloroform and sodium hydroxide extraction and sample clean-up using solid phase extraction (SPE; Rottinghaus et al., 1991; Rottinghaus et al., 1993). The HPLC method was routinely used as the standard method for analyzing ergovaline levels in fescue plant tissue (Schnitzius et al., 2001) with ergotamine as an internal standard, which is typically produced by plant endophytes in significantly low quantities (Yates et al., 1985; Rottinghaus et al., 1991). Chromatographic methods with fluorescence detection sufficient for the quantification of ergometrine, ergotamine, ergocornine, L-ergocryptine and ergocristine at levels as low as 2 - 5 ng/g was used for direct monitoring of ergot alkaloids in food and feed commodities (Wolff et al., 1988). Additionally, this analytical method has been validated for twelve different ergot alkaloids in grain and flour (Müller et al., 2009).

Furthermore, an HPLC assay developed to detect and quantify the smaller ergot alkaloids like ergolines in complex fluids showed recovery of > 80%, especially in ruminal fluid, urine, and feces (Lodge-Ivey et al., 2006). Combining HPLC with fluorometric detection has also been shown to be an effective method to analyze ergovaline in plasma and milk (Jaussaud et al., 1998; Durix et al., 1999). Ergovaline is the predominant ergot alkaloid, comprising nearly 84 to 97% of the ergopeptide alkaloids in tall fescue infected with *Epichloë* (Lyons, 1986; Bush et al., 1997), with concentrations as high as 14 $mg \cdot kg^{-1}$ in sheaths and 1.5 $mg \cdot kg^{-1}$ in blades. Lysergic acid and its amides (i.e. ergine and ergonovine) can be present in concentrations similar to that of ergovaline (Porter, 1995) and concentrations of these have been shown to increase quadratically with the increase in the environmental temperature (Salminen et al., 2005). Even though HPLC has been the preferred method for analysis of ergot alkaloids, it does not differentiate compounds having a very narrow window in molecular weight and molecular structure (Di Mavungu et al., 2012) which tend to co-elute during chromatographic separation (Kokkonen and Jestoi, 2010). Therefore, researchers started using LC-MS/MS to analyze ergot alkaloids in a single sample run by differentiating the molecules based on molecular weight.

In recent decades, ultra-high performance liquid chromatography - mass spectrometry (UPLC-MS) has been the choice of instrument for analysis of compounds of interest that are different in molecular weight, even within small margins. Ergot alkaloids are separated by UPLC and ionized in an electrospray (ESI) to a protonated molecular ion. The ionized molecules are further fragmented under the collision gas in the subsequent step into characteristic charged product ion molecules (Lehner et al., 2004), which are then separated and detected in multiple stage mass spectrometry (Lehner et al., 2005; Mohamed et al., 2006). In LC-MS/MS quantitative analysis, the monitoring of relatively intense and definite fragment ions is used to confirm the identity of the ergot alkaloids and for quantification (Lehner et al., 2004). During fragmentation of ergot alkaloids, a cleavage of the amide bond of ergopeptides or the ether groups of ergoline ring at the C-12' alpha-hydroxy group leaves behind a fragment of m/z 320 (Arroyo-Manzanares et al., 2014). Specific transitions typically used includes $[M+H]^+ \rightarrow$ m/z = 268, 251, 225, 223, and/or 208 (Krska et al., 2008b) which can arise from successive losses of NH_3, CO, and/or CH_3. Other ergopeptides including ergocornine, ergocryptine and ergocristine with m/z 348 were observed, indicating retention of the isopropyl group of the fragment (Reinhard et al., 2008). Transitions of the protonated precursor ion (M+H+) to m/z 223 are frequently used as the quantifier ion with the transitions to m/z 208 as a qualifier. Validation of mass spectrometric

methods has been carried out by several researchers, quantifying most prevalent mycotoxins and ergot alkaloids using internal standards (Lehner et al., 2005; Krska et al., 2008; Di Mavungu et al., 2012; Jackson et al., 2012).

With LC-MS/MS detection, targeted mass analysis is usually conducted, where a few selected masses can be monitored, while time of flight mass spectrometry detection (LC-TOF-MS) provides the information of whole mass range in the analyte. Using LC-TOF-MS, spectral information for a large number of compounds can be extracted from a single LC run that provides high mass resolution with a low level of background noise and the associated high sensitivity better signal: noise ratio. In addition, a third detection method, quadrupole-time-of-flight mass spectrometer (LC-QTOF-MS) provides more specificity, sensitivity and detailed results with molecular weights discriminated at up to four decimal points. Application of LC-QTOF-MS to determine ergot alkaloids in rye extracts cleaned up using an immobilized aptamer showed the presence of ergosine at (m/z 547.2874) and ergocryptine (m/z 575.3085) using a characteristic precursor fragment at m/z 223.1283 (Rouah-Martin et al., 2014).

Even though LC-MS/MS provides better analytical information, one of the drawbacks in mass spectrometric detection following LC is the alteration of the signal intensity due to co-extracted compounds that affect the ionization efficiency—so-called matrix effects. Matrix effects lead to either signal enhancement or suppression which is usually addressed to some degree by matrix-matched calibration (calibration standards prepared in a matrix extracted from a “blank” sample). Additionally, matrix effects can be addressed to a large extent through the use of isotopically labeled internal standards, after making sure that analyte and isotopically labeled analyte have similar signal interferences from the matrix. Deuterated analogs (d3-, d6- and d10-LSD) are available for analysis of lysergic acid diethylamide, which is most commonly used in forensic applications (Musshoff and Daldrup, 1997; Berg et al., 2013). The use of internal standards is more common when those standards are commercially available. For example, methysergide maleate has been used as an internal standard in the analysis of six major ergot alkaloids in human cells (Mulac and Humpf, 2011). Dihydroergocristine has been used as internal standard in the analysis of serum dihydroergotamine (Romeijn et al., 1997) and in the determination of 16 different alkaloids in bread, flour and infant formula (Reinhard et al., 2008). A systematic study has been conducted to determine the matrix effects for a range of cereals in the LC-MS/MS analysis of ergot alkaloids with the aim of eliminating or reducing their effect

(Malysheva et al., 2013). Different cereals and different varieties of cereals showed different matrix effects, where a 90% suppression was observed for ergometrine in barley and oats and a 50% suppression was observed for ergocryptinine and ergocristinine in those cereal grains. Therefore the blank matrix material used for the preparation of matrix matched standards must be carefully chosen.

2.8. Summary

The adverse effects of endophyte-infected tall fescue on livestock have been well established in the last few decades. Consumption of endophyte-infected tall fescue by cattle results in numerous physiological changes. Despite a tremendous amount of research and common use of practices designed to reduce the impact of fescue toxicosis, current strategies falls short of eliminating the toxic effects. One practical approach is the use of feed additives that utilize adsorbent technology to reduce bioavailability of alkaloids in the gut and decrease the incidence of ergot alkaloids entering the blood stream. Yeast cell wall and clay-based adsorbents have found widespread use for reduction of mycotoxin bioavailability. Some adsorbents are selective and specific toward certain classes of toxins, while some adsorbents are non-specific. Yeasts and their cell wall components are used as adsorbents that effectively reduce mycotoxicosis in livestock. Freimund and co-workers (2003) showed that crosslinked 1,3-β-d glucan modified by carboxymethyl ether and hexadecyltrimethylammonium salt demonstrated the highest ability to bind zearalenone (183 mg/g) and T-2 toxin (10 mg/g). The application of β-D-glucans from yeast cell wall as specific adsorbent for zearalenone have been well established (Yiannikouris et al., 2004; Yiannikouris et al., 2006). Modified clay with polymeric Al/Fe species have shown specific binding towards humic acid compared to unmodified clay (Jiang and Cooper, 2003). Another class of target specific adsorbent is imprinted polymers that were developed in the late 90s using molecular imprinting technology. The use of imprinted polymer adsorbents has been mainly in medical and analytical applications as an alternate to bio-receptors and chemical adsorbents. The use of imprinted polymers as an adsorbent in animal feed is a novel idea and will be beneficial in the event that imprinted polymers synthesized using compounds representative of alkaloids (structurally and functional groups) can mitigate the toxic effects caused by a family of ergot alkaloids.

There is limited data related to the synthesis of imprinted polymers for ergot alkaloids and their utility in SPE and animal feed as adsorbents. For application as a feed supplement for

ruminant animals, the class of livestock which most commonly encounter ergot toxicity, the addition of polymer in the diet needs to have minimum or no negative effects on ruminal fermentation. In addition, the binding characteristics generated for the imprinted polymer from *in vitro* studies need to be evaluated for the intended use. To date, researchers have not dealt with determining the significance of *in vitro* binding parameters generated from isothermal adsorption studies. It is this gap that the research presented in this dissertation aims to fill. Apart from synthesizing imprinted polymers, the research presented here attempts to characterize the morphology and binding characteristics of the synthesized polymers and to evaluate key aspects related to the intended use of the products, either as a potential feed additive or as an adsorbent material for application in analytical chemistry to facilitate ergot alkaloid measurement in feed and other biological samples.

Table 2-1. Lethal dose (LD_{50}) values for different ergot alkaloids in rabbits, rats and mice after intravenous (i.v.) or oral application data from (Rutschmann et al., 1978)

		LD_{50} (mg/kg body weight)					
		Rabbit		Rat		Mouse	
Alkaloid		i.v.	oral	i.v.	oral	i.v.	oral
Lysergic acid derivatives	d-LSD	0.31	n.s.	n.s.	n.s.	n.s.	n.s.
	Ergonovine (−metrine)	3.20	28	120	671	160	460
Ergopeptide derivatives	Bromocriptine	12.0	n.s	n.s.	n.s.	n.s.	n.s.
	Ergocornine	0.90	n.s.	95	>500	275	2000
	Ergocristine	1.90	n.s.	64–150	n.s.	110	n.s.
	Ergosine	1.23	n.s.	n.s.	n.s.	n.s.	n.s.
	Ergotamine	3.0	550	38	1300	265	3200
	Ergovaline	1.70	n.s.	n.s.	n.s.	n.s.	n.s.
	l-Methylergotamine	21.0	n.s.	n.s.	n.s.	n.s.	n.s.
	α-Ergocryptine	1.0–0.8	n.s.	140	n.s.	275	n.s.

n.s. not specified

Table 2-2. Scatchard, Langmuir and Freundlich Adsorption isotherm models to determine adsorption parameters (affinity, capacity and favorability of adsorption)

Model	Equation	Parameters
Scatchard	$\frac{B}{[F]} = \frac{B_{max} - B}{K_D}$	B is the amount of template bound to polymer at equilibrium, µmol·mg^{-1} [F] is the concentration of free template in the solution, µmol·L^{-1} B_{max} is the apparent maximum number of binding sites, µmol·mg^{-1} K_D is the apparent dissociation constant
Langmuir Linear form	$qe = \frac{Q_o\, K_L\, C_e}{1 + K_L\, C_e}$ $\frac{1}{qe} = \frac{1}{Q_o} + \frac{1}{Q_o\, K_L\, C_e}$ $R_L = \frac{1}{\left(\frac{1}{1 + K_L\, Co}\right)}$ $\Delta G^o = RTln(55.5K_L)$	q_e is amount adsorbed, µmol·mg−1 K_L is Langmuir adsorption constant Q_o is maximum amount adsorbed (µmol ·mg^{-1}) C_o is initial concentration of adsorbate (µmol·L^{-1}) C_e is equilibrium concentration of adsorbate (µmol.L^{-1}) Langmuir isotherm constant R_L (favorability of adsorption). 0<R_L<1 favorable, R_L>1 unfavorable, R_L=1 linear, R_L=0 Irreversible ΔG_o is Gibbs free energy R is gas constant (3\8.314 J•mol-1•K-1 T is the absolute temperature in Kelvins
Freundlich Linear form	$qe = K_f C_e^{\left(\frac{1}{n}\right)}$ $Log\ qe = logK_f + \left(\frac{1}{n}\right) log\ Ce$	K_f : Adsorption capacity 1/n: adsorption intensity or surface heterogeneity. 1<n<10: favorable adsorption (1/n < 1) 1/n > 1: Co-operativity in adsorption n = 1: Interaction between the two phases are independent

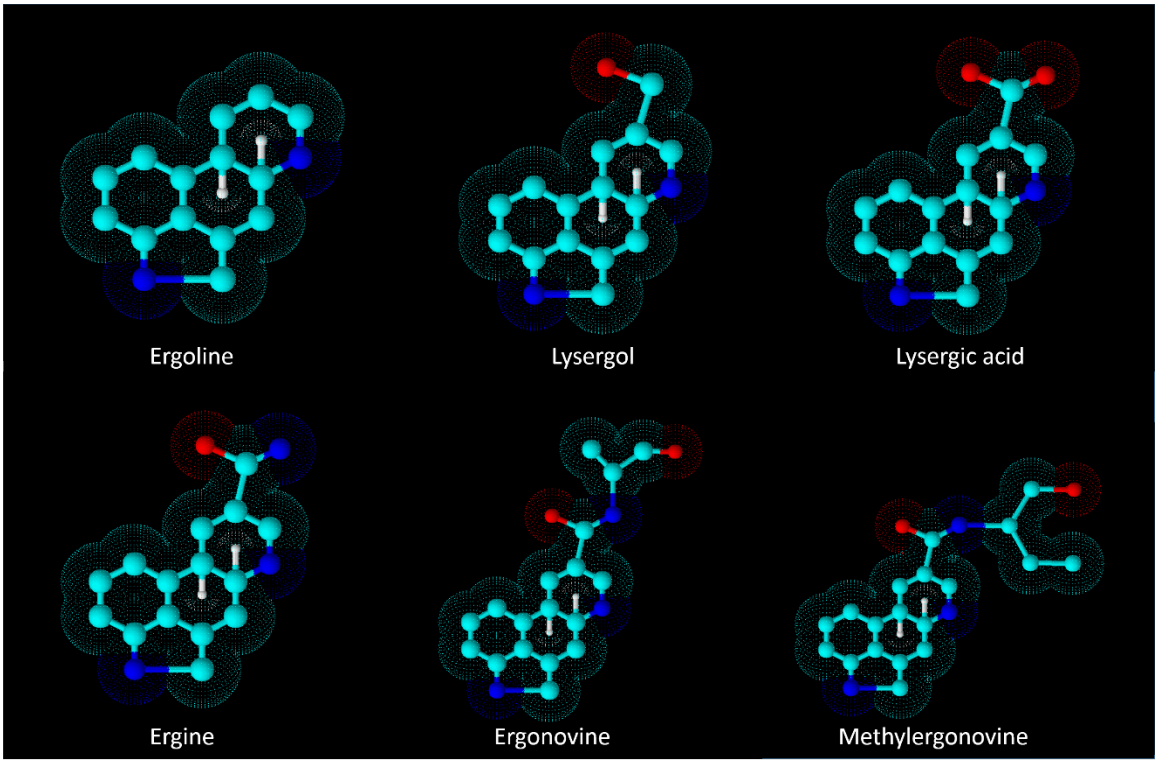
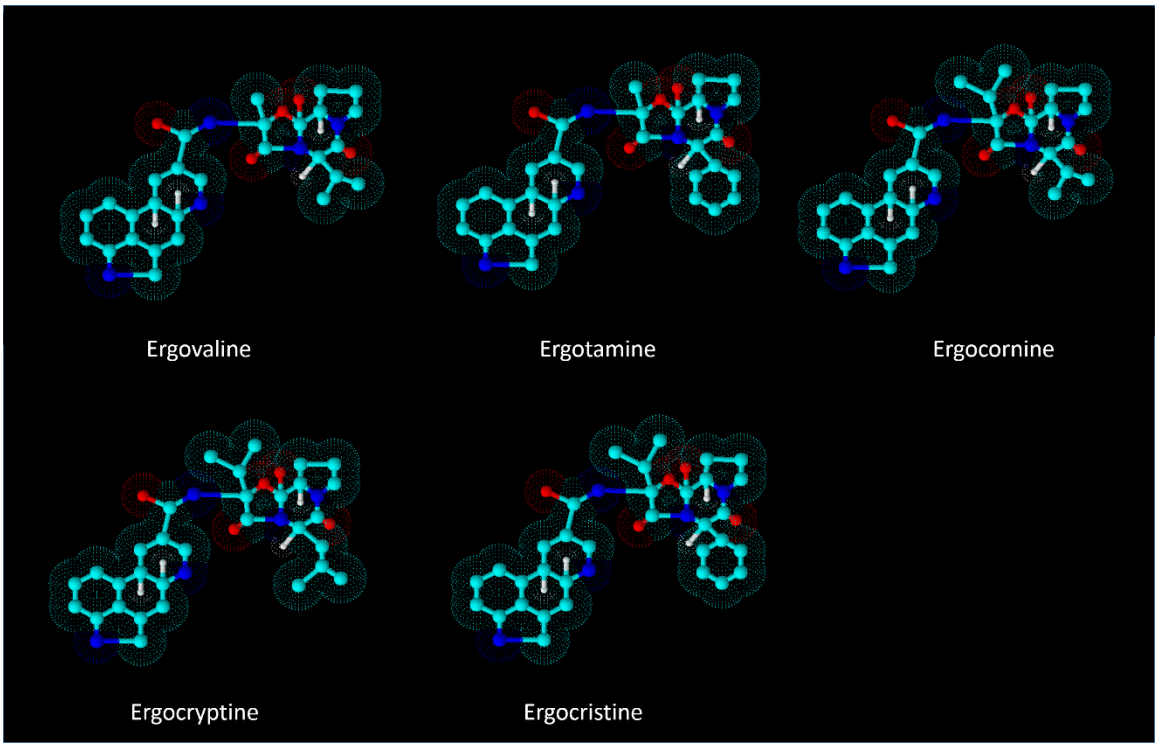


Figure 2-1. Structure of ergoline and its derivatives at C-8 position



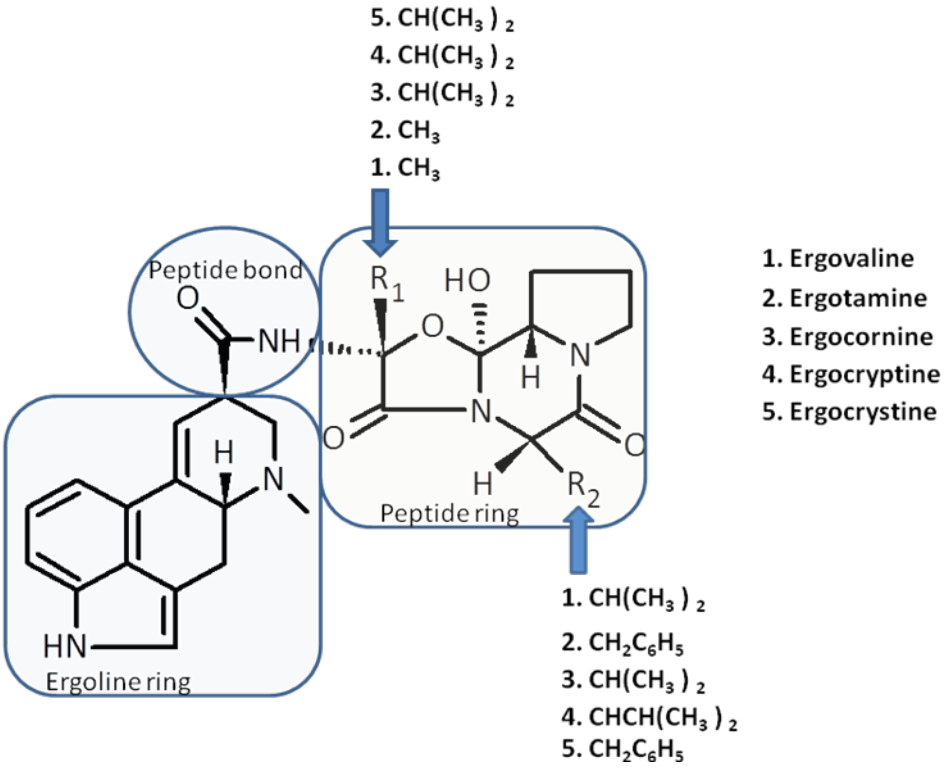


Figure 2-2. Structure of ergopeptide derivatives that have physiological significance.

3. SYNTHESIS, EVALUATION AND CHARACTERIZATION OF A STYRENE BASED ERGOTAMINE IMPRINTED POLYMER FOR POTENTIAL USE AS AN ERGOT ALKALOID HIGHLY SELECTIVE ADSORBENT

3.1. Introduction

Tall fescue (*Lolium arundinacea*) is a cool season perennial grass widely used as forage in the eastern and northwestern United States. The popularity of tall fescue is mainly due to its ability to withstand extreme stress conditions. Animal industries in areas dominated by fescue have suffered significant financial loss due to fescue toxicosis that is caused by a family of alkaloids produced in endophyte infected tall fescue (Hoveland, 1993). Ergot alkaloids are secondary metabolites produced by the endophyte *Epichloë coenophiala*, a symbiotic fungus of tall fescue (*Festuca arundinacea*) which helps the grass resist abiotic (extreme weather) and biotic (repel nematodes) stresses (Rodriguez et al., 2009). Ergot toxicities, including "fescue toxicosis" have been reported in animals grazing endophyte tall fescue (Schmidt et al., 1982; Strickland et al., 1993), and "rye grass staggers" in sheep consuming *Acremonium lolii* infected ryegrass. Chemically, ergot alkaloids consist of an ergoline ring and its amine derivatives with two or more functional groups (Mantegani et al., 1999). Toxicity arises as a consequence of binding of α1 and α2 adrenergic, D_2 dopaminergic and a family of 5HT2 serotonergic receptor sites (Rowell and Larson, 1999; Gornemann et al., 2008; Foote et al., 2011b; Strickland et al., 2011). Toxicological effects range from weight loss in mild cases to death in severe cases depending on the environmental stress factors and level of exposure to alkaloids (Mulac and Humpf, 2011b; Strickland et al., 2011). Toxicity symptoms become more severe when co-occurrence of different alkaloids are encountered. However, in the majority of cases, ergovaline has been reported as the most abundant (Repussard et al., 2014) alkaloid in endophyte-infected tall fescue and is considered the putative cause for most alkaloid toxicity. Due to the extent of financial loss (> $1 billion; Strickland et al., 2011) and the dependence of animal industries on fescue, monitoring and mitigation strategies are needed to control animal exposure to such contaminants.

Monitoring contamination levels of ergot alkaloids can be achieved through high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) with simultaneous quantification of major alkaloids as well as their epimers at low concentrations (Diana et al., 2012). The LC-MS/MS method works on the principle of target molecule ionization and

fragmentation and the sensitivity in analysis can be enhanced in the absence of ion suppression or ion enhancement by using the sample matrix for standard analysis (Fureya et al., 2013) and by reducing interfering compounds (Matuszewski et al., 2003; Fureya et al., 2013). The accuracy and precision of analysis can be dramatically improved by using purification strategies to mitigate the impact of the sample matrix and/or the necessity of internal standards. Several sample clean-up techniques have been adopted, including the use of liquid-liquid extraction (Billups et al., 2010), immune-affinity columns (Webb et al., 1996), filtration, centrifugation, and solid phase extraction (SPE). However, the SPE technique is gaining prominence due to its selectivity and recovery of analyte with the advent of new SPE materials called molecularly imprinted polymers (MIP; Lenain et al., 2012a).

Molecular imprinting is a technique for synthesizing macromolecular polymers (molecularly imprinted polymers; MIP) with specific binding pockets and multi-functional receptor groups that can form complexes with the targeted molecule(s) (Karsten and Klaus, 2000; Sellergren and Andersson, 2000; Lian et al., 2015). Molecular imprinting technology deals with template-based, pre-organized cavities with high binding affinity for a specific template and closely related molecular species (Rampey et al., 2004). The complementary structural and molecular functionalities between the imprinted polymer and the targeted template molecule govern the specificity of its molecular recognition properties. Specificity of an MIP for a template has been utilized in extraction and removal of impurities in water waste management (Sun et al., 2008), drug delivery (Barde et al., 2013), and in a range of biotechnological applications.

An imprinted polymer towards lysergic acid diethylamide (LSD) that was synthesized using methacrylic acid (MAA) functional monomers and ergometrine as a template (Chapuis-Hugon et al., 2009) showed 82% extraction recovery of LSD analogs from hair and urine samples. Similarly, an MIP with MAA monomer cross linked with ethylene glycol dimethacrylate (EGDMA) in chloroform that was imprinted with metergoline template exhibited high selectivity towards the template when compared to non-imprinted polymers (NIP; Lenain et al., 2012). However, cross reactivity with certain polycyclic compounds occurred with both MIP and NIP. Most imprinting studies have used acrylates as the functional monomer and studies using styrene based imprinted polymers, especially for ergot alkaloids, are limited.

The goal of the present study was to develop an ergotamine-imprinted styrene-hydroxyethyl methacrylate (HEMA)-based MIP that can interact with different ergot alkaloids possessing common ergoline ring structures. This report details the morphological and physical characteristics and template rebinding of molecularly imprinted copolymers using commonly used adsorption isotherm models. This work also aimed at identifying the nature of the molecular interactions between the polymer and template to understand specificity, selectivity and the binding site properties. Such solid phase binding materials may have utility in extraction and clean-up of complex feed or fluid matrixes, especially adaptable to ruminant feeds.

3.2. Material and methods

3.2.1.Chemicals

Ethylene glycol dimethacrylate (EGDMA, ≥97%), 2-hydroxyethyl methacrylate (HEMA, >98%), 1,1,3,3-tetramethylguanidine (TMG, >97%), styrene (≥99%), and 2,2-azobisisobutyronitrile (AIBN, ≥98%) were purchased from Fluka (Sigma-Aldrich, Milwaukee, WI). 2-bromo-α-ergocryptine methanesulfonate (BC), methylergonovine maleate (ME), ergotamine-D-tartrate (ETA) and lysergol (LY) were purchased in purified crystalline form (≥ 97%) from Sigma-Aldrich (St. Louis, MO). Solvents used in polymer synthesis and analytical methods, including methanol (HPLC-grade), acetonitrile (optima grade), acetic acid and formic acid (reagent grade), were purchased from Fisher Scientific (Fair Lawn, NJ). High purity water used in all experiments was obtained from a Milli-Q Ultra-pure water purification system (Millipore Corporation, Bedford, MA).

The fungal mycotoxin standards: cyclopiazonic acid (CPA), aflatoxin B1 (AF B1), ochratoxin A (OTA), deoxynivalenol (DON), fusaric acid (FA), zearalenone (ZEA), roquefortine C (Roq C) and sterigmatocystin (STG) were purchased from Sigma−Aldrich (St. Louis, MO). Diacetoxyscirpenol (DAS) was purchased from Biopure (Tulln, Austria). Individual standard stock solutions of mycotoxins (1 mg·mL^{-1}) were prepared in methanol and stored at -20 $^{\circ}$C in the dark until use. Working solutions of studied compounds were prepared from individual stock solution by dilution with 0.1 M phosphate buffer of pH 6.8.

3.2.2.Molecularly imprinted polymer synthesis and characterization

3.2.2.1. Synthesis of MIP

Imprinted polymers were synthesized by self-assembly bulk polymerization using ETA as the molecular template, styrene and HEMA as functional monomers, EGDMA as the crosslinker, AIBN as free radical initiator and toluene as the porogen. Ergotamine tartrate was neutralized by adding 0.6 g of TMG during polymer synthesis to generate the free base form of ETA. Solutions including styrene, HEMA and EGDMA were distilled under vacuum prior to use to remove inhibitors.

Ergotamine D-tartrate (0.005 moles) was dissolved in 10 mL of methanol containing 0.005 moles of TMG in a 250 mL triple neck borosilicate round bottom reactor. Monomers including styrene (0.08 moles) and HEMA (0.04 moles) were added and mixed for 30 min. After mixing, toluene (75 mL) and cross linker (EGDMA: 0.1 moles) were added. Nitrogen was purged through the solution throughout the entire procedure. The mixture was heated in an oil bath to 65 $^{\circ}$C and AIBN was added to initiate polymerization and the formation of the MIP. The corresponding non-molecularly imprinted polymer (NIP) was synthesized using the same procedure without ETA template. The polymerization reaction was stopped after 5 h and the polymer was subjected to a template washing procedure. To remove template, polymer mixtures were filtered through a 0.2 μm filter (Celite 577^{TM}, World Minerals Inc. Santa Barbara, CA) and the filtrate was collected to analyze for ETA concentration. Template molecules trapped in the polymer matrix were washed using ten successive acidic methanol washes (0.2 N HCL in methanol) followed by two buffer washes (0.1 M sodium citrate buffer of pH 4) and a DI water wash. Five washes were conducted on the first day and four washes on each of the subsequent two days. The polymers (MIP and NIP) were left in the wash solvent overnight and continued the wash procedure the next day. Due to incomplete recovery of template from MIP in initial washes, five subsequent washes each in methanol and acetonitrile were performed, again followed by a DI water wash. The polymers were considered complete when the bleeding of ETA was reduced to negligible levels as confirmed by UPLC-MS/MS analysis of washings.

Each template wash procedure was carried out with 100 mL of washing solvent with vigorous shaking (15 min) and sonication (15 min) followed by centrifugation (7263 g for 30 min) and filtration of supernatant. Filtrates were analyzed for ETA concentration to check for total

recovery of template. After template removal, the polymers were freeze-dried (<100 mT, – 46 °C for 48 h), oven-dried (60 °C overnight) and weighed. The dried polymer was ground to fine powder using a mortar and pestle and sieved using standard metal sieves (VWR- USA Standard Testing Sieves) to obtain a < 250 µm particle size fraction.

3.2.2.2. Morphological characterization

3.2.2.2.1. Nitrogen sorption porosimetry

Structural properties of polymers were characterized using nitrogen adsorption/desorption isotherms at -196.15 °C according to the Brunauer-Emmett-Teller (BET) procedure (Brunauer et al., 1938). Polymers were evaluated for pore size distribution, pore volume and specific surface area. A 15.0 mg sample was heat treated (100 °C) for 3 hours under inert gas flow to remove atmospheric contaminants. The sample was then cooled, degassed under vacuum (1 x 10^{-5} Torr) and exposed to increasing nitrogen gas pressures. Nitrogen gas adsorption/desorption was measured under cryogenic conditions using an Autosorb-1C (Quantachrome Instruments, FL, USA) gas sorption analyzer. The BET surface areas were calculated from the adsorption isotherms in the relative pressure range from 0.06 to 0.2 psi. Pore size distribution and total pore volume were determined using the Barrett-Joyner Halenda (BJH) method (Barrett et al., 1951).

3.2.2.2.2. Microscopy

Electron microscopy was performed on MIP and NIP to investigate surface morphologies. A field emission scanning electron microscope (TEM: JEOL 2010F, JEOL, Tokyo, Japan) operating at 200 kV was used to monitor the topography of polymers. As sample preparation procedure, the polymer (5 mg) was suspended in methanol (10 mL), sonicated (35 °C for 10 min), dispersed on a carbon-coated microscopic copper grid (200 mesh size) and dried under vacuum at room temperature. Additional morphological characteristics were investigated by scanning electron microscopy (SEM: Hitachi S-4300, Tokyo, Japan). Polymer samples were spread on carbon tape and sputtered with gold (Emscope SC400 Quorum technologies Ltd. East Sussex, UK) before loading onto an aluminum disc. Samples were exposed to the desired voltage (15 keV) and current beam under an aperture width of 2.80 µm with automatic filament saturation.

3.2.2.2.3. Dynamic Light Scattering (DLS)

The hydrodynamic diameters of the polymers were determined by dynamic light scattering (DLS) measurements. Polymers were suspended in double distilled water (0.1 mg·mL^{-1}) or in 5% aqueous methanol and sonicated (1 min) at room temperature. Particle size distribution was measured using continuous wide angle dynamic light scattering detection (SALD- 7101, Nanoparticle size analyzer, Shimadzu Scientific instruments, Columbia, MD) using a UV laser as the light source (375 nm). The instrument was set to make 15 measurements between 0.5 nm and 300 µm under automatic mode and diffraction data were obtained as the volume percentage of particle versus the particle size. A reference spectrum of the solvent under similar conditions was used to correct for background noise.

3.2.2.3. Adsorption studies

3.2.2.3.1. Isothermal adsorption

Isothermal adsorption studies using polymers and template were conducted in equilibration media consisting of pH 6.8, 0.1 M phosphate buffer. One mg polymer was exposed to increasing concentrations of ETA, (C_o: 0.00, 0.15, 0.76, 1.52, 7.61, 15.23 µmol·L^{-1}), in 10 mL equilibration media (*V*). Samples were incubated on a horizontal shaker at 39 °C for 90 min and then centrifuged (14500 x *g*, 10 min) to separate the free ETA from ETA-polymer complex according to the protocol described for equilibrium experiments (Lenain et al., 2012). The supernatants were analyzed for ETA (C_e, µmol·L^{-1}) by high pressure liquid chromatography fluorescence detection (Alliance, Waters Corp., Milford, MA). The amount of ETA adsorbed (q_e) per unit of polymer (*W*) was determined by the difference between the control and supernatant ETA concentrations as shown below

$$q_e = (C_o - C_e)V/W,$$

where q_e is the amount adsorbed (µmol·mg^{-1})

V is the volume used (mL)

C_o and C_e are initial and equilibrium concentrations (µmol·L^{-1}) of ETA, respectively

W is the weight of polymer (mg)

The adsorption properties of polymers were evaluated by nonlinear regression models including Langmuir and Freundlich (Table 3-2). Isothermal adsorption parameters from the Langmuir model including adsorption constant (K_L), maximum monolayer adsorption capacity of the adsorbent (Q_o) and adsorption favorability constant (R_L), and Freundlich adsorption parameters including extent of adsorption (K_f) and surface heterogeneity or adsorption intensity (n) were determined (GraphPad Prism Software, version 5.0, USA or Datafit software, Datafit version 8.1.69, Oakdale engineering, Oakdale, PA USA). The fits were ascertained using correlation coefficients and lowest residual variance. The model that described the adsorption parameters was selected based on lowest absolute sum of squares of residuals or the probability of fit for one model versus the other that was determined using bias-corrected Akaike's Information Criterion (AICc) for the two models (GraphPad Software Inc., La Jolla, CA)

3.2.2.3.2. Selectivity

To evaluate the selectivity of MIP for ETA, a study was conducted in 0.01 M ammonium citrate buffer at pH 6.7, containing structurally related alkaloids. A mixture of ergot alkaloids (15ppm) (Figure 3-1; ETA: 11.42 μmol·L^{-1}, BC: 19.98 μmol·L^{-1}, ME: 32.93 μmol·L^{-1} and LY: 58.97 μmol·L^{-1}) was prepared in 10 mL buffer and serially diluted (5x) by a factor of two to obtain six levels of mixture. Also, a control solution was prepared without alkaloids. Polymers (0.1 mg·mL^{-1}) were added to increasing concentrations of alkaloid mixtures and incubated for 90 min at 39 °C. The samples were centrifuged (14,500 x *g*, 10 min) and the supernatant was analyzed for alkaloids using UPLC-MS/MS. Selectivity of each alkaloid was evaluated using the adsorption coefficient (*k*) of each alkaloid calculated as:

$$k = \frac{[Co - Ce]}{M} \times V$$

where, *Co* and *Ce* are the initial and final equilibrium concentrations of ergot alkaloids (μmol·L^{-1}), *V* is the volume of adsorption medium in liters, and *M* is the amount of polymer used in the medium in grams. Selectivity for the adsorption of ETA in the presence of interfering compounds was estimated by the selectivity coefficient (*k'*) using the equation:

$$k' = \frac{k(ETA)}{k(alkaloids)}$$

where, alkaloids are represented by BC, ME and LY. The effect of imprinting on selectivity (*k''*) was determined by the ratio of selectivity coefficients of imprinted to non-imprinted polymer within each alkaloid.

$$k'' = \frac{k'\ imprinted}{k'\ non-imprinted}$$

3.2.2.3.3. Cross-reactivity

Cross reactivity of the polymers with structurally diverse mycotoxins was evaluated. A working solution was prepared with an increasing concentration of different toxins (cyclopiazonic acid (CPA), aflatoxin B1 (AF B1), ochratoxin A (OTA), deoxynivalenol (DON), fusaric acid (FA), diacetoxyscirpenol (DAS), zearalenone (ZEA), roquefortine C (Roq C) and sterigmatocystin (STG)) from individual stock solutions (1 g·L^{-1} in methanol) in 0.01 M ammonium citrate buffer of pH 6.7. A 9 mL working solution at each concentration was individually mixed with 1 mL volume of adsorbent slurry (1 g·L^{-1}) in triplicate. The initial mycotoxin range was chosen based on natural levels of contamination (Placinta et al., 1999; Binder et al., 2007) and to provide detectable concentrations assuming 95% adsorption. The calculated mycotoxin range included: ETA and FB1 of 0.0625 – 1.5 mg·L^{-1}; CPA of 12.5 – 300 µg·L^{-1}; AFB1 and OTA of 7-150 µg·L^{-1}; DON and FA of 42-978 µg·L^{-1}, T-2, DAS and ZEA of 14-334 µg·L^{-1}; RoqC of 4-100 µg·L^{-1} and STG of 11-250 µg·L^{-1}. Along with the samples, three controls were prepared: a buffer control containing no toxin and no adsorbent, a toxin working solution control at each concentration, and a control containing only a single adsorbent. Samples and controls were capped and incubated with agitation (150 rpm and 39 °C) for 90 min. After incubation, the samples were centrifuged (14,500 x *g*) for 10 min to separate the polymer-toxin complex from the free toxin. The supernatant was analyzed for all toxin concentrations using UPLC –MS/MS. The amount of mycotoxin adsorbed was determined by the difference in concentration between the control and the test sample with same initial loadings.

3.2.2.3.4. Effect of pH and temperature

To determine the effect of pH, an adsorption study was conducted in 0.1 M potassium phosphate buffer (pH 2, 3, 4, 5, 6, 7, 8, 9 and 10), at an ETA concentration of 1 mg·L^{-1} and polymer concentration of 0.1 mg·mL^{-1}. The samples were incubated for 90 min at 39 °C on a horizontal shaker (150 rpm) and centrifuged (14,500 x *g*, 10 min) to separate the adsorbent-

toxin complex from the free toxin. The supernatant was analyzed for free ETA by LC-MS/MS and the adsorption was calculated by taking the difference between the controls and the test samples.

The effect of temperature was determined by adsorption studies at 36, 39 and 42 °C. Polymer (0.1 $mg \cdot mL^{-1}$) was exposed to 1 $mg \cdot L^{-1}$ ETA in 10 mL of 0.1 M potassium phosphate buffer (pH 6.8) at each temperature for 90 min on a horizontal shaker (150 rpm). Samples were centrifuged (14,500 x *g*, 10 min) to separate the adsorbent-toxin complex from the free toxin. The supernatant was analyzed for free ETA, by HPLC-FLD and adsorption was calculated as the difference in supernatant ETA concentration between controls (no polymer) and test samples.

3.2.2.3.5. Adsorption in rumen fluid solution

The adsorption efficiency of different levels of MIP to ETA in comparison to control (NIP) in rumen fluid was determined to evaluate the effect of a complex matrix on adsorption. Ruminal fluid was collected from fistulated steers grazing on endophyte-free fescue pasture. The animals were gathered from the pasture two hours before collection of rumen fluid. Approximately 1.5 to 2 L of ruminal fluid was collected and filtered through 4 layers of cheesecloth into a warm (39 °C) 1 L thermos flask. Ruminal fluid was then autoclaved (121 °C, 30 min, 15 psi) and centrifuged (7263 x *g*, 30 min) to remove the sediments. For adsorption studies, 500 mL of rumen fluid was diluted with 500 mL of McDougall's buffer (McDougall, 1948).

A known concentration of ETA (.0033 $mg \cdot mL^{-1}$) was exposed to increasing concentrations of polymer (0.001, 0.01, 0.1, 1 and 2 $mg \cdot mL^{-1}$) in 100 mL of rumen fluid. Increasing concentration of polymer in ruminal fluid was prepared by a slurry technique, where a stock solution of polymer (20 $g \cdot L^{-1}$ rumen fluid) was prepared and diluted in rumen fluid to the required concentration. The stock slurry solution was continuously mixed while dilutions where prepared. The rumen fluid samples were then spiked with 0.33 µL of ETA stock solution (1 $mg \cdot L^{-1}$) to obtain concentration of 3.3 $mg \cdot L^{-1}$. The samples were incubated for 90 min with agitation (150 rpm, 39 °C), centrifuged (14500 x *g*, 10 min) and the supernatants were analyzed for ETA concentration by HPLC-FLD.

3.2.2.4. Fourier transform infrared analysis

A Perkin-Elmer 100 spectrometer was used for Fourier transform infrared (FTIR) analysis of polymers (MIP and NIP) and polymer-template complexes to determine the functional groups involved in interactions according to procedures described for polymer interactions (Molinelli et al., 2005). The spectrometer was equipped with an overhead ATR diamond crystal that was cleaned with alcohol between measurements. A 10 mg sample was placed on the diamond crystal and absorbance was recorded in the range 4000 to 600 cm^{-1} for qualitative examination of the vibrational frequencies of functional groups such as C-C, C-H, C=C, O-H and N-H. For each freeze dried sample, 10 scans with resolution of 2 cm^{-1} with automatic baseline correction were performed.

3.2.3.Analytical method

Detection and quantification of ergot alkaloids and mycotoxins were performed with a UPLC-ESI-TQD MS/MS system (Acquity, Waters Corp., Milford, MA, USA) fitted with an electrospray ionization source and running in positive ion mode (ESI+). Analyses were performed by means of a multi-reaction monitoring experiment (MRM) targeting analytes (parent and daughter ions) within their specific retention window. Chromatographic separations were performed using 1.7 µm, Acquity ethylene-bridged C_{18} hybrid particle columns (2.1 mm x 100 mm, Waters Corp) maintained at 40 °C. A two-solvent gradient mobile phase composed of water (eluent A) and methanol (eluent B), both acidified with 0.1% formic acid was used at a flow rate of 0.42 $mL \cdot min^{-1}$ and a 10 µl of sample was injected using an auto-sampler. The elution gradient started at 5% B for the first 2 min followed by a linear increase to 10% B for 2 min, then to 75% B for the next 8 min, with maintenance at 99% B for 2 min, and finally re-equilibration for 2 min with 5% B. Nitrogen (Nitroflow, Parker-Balston, Haverhill, MA, USA) was used as the desolvation and cone gas (Jackson et al., 2012).

A series 2695 Alliance HPLC separation module (Waters, Milford, MI) equipped with a binary pump, an auto sampler, column oven and a fluorescence detector (474 scanning fluorescence detector, λ_{ex} 250nm, λ_{em} of 420nm, gain 16 and attenuation 1000) was used to detect ergot alkaloids in the samples. Chromatographic data were integrated using Waters Empower 3 software 7.00.00.99 (Waters, Milford, MI). HPLC separations were performed with a 100 x 4.6 mm i.d., 2.6 µm particle size, Kinetex C_{18} column (Phenomenex, Torrance, CA) with a

gradient elution consisting of two mobile phases, A) water and B) acetonitrile, both spiked with ammonium hydroxide (0.04%). Initial gradient conditions were 100% A held for 1 min, increasing linearly to 100% B over 12 min and held for 3 min. The final step was a linear return to initial conditions over 3 min, which was then held for 2 min for a total run time of 23 min. The sample injection volume was 50µL. Samples were evaporated to near dryness and the residue reconstituted in methanol/water (50/50) and analyzed (Rottinghaus et al., 1991).

3.2.4.Statistical analysis

Results of *in vitro* adsorption studies were analyzed with the GLM procedure (SAS Inst. Inc., Cary, NC). Triplicate samples were averaged for statistical analysis. The model included concentration of analyte, type of analyte, adsorbent concentration, and the interaction of analyte concentration × type of analyte. Least squares means were calculated for analyte concentration, type of analyte, and the analyte concentration × type of analyte interaction. The adsorption models were compared using AICc for the best fit model (Spiess and Neumeyer, 2010) using the option in GraphPad (GraphPad Prism Software, version 5.0, USA).

3.3. Results and discussion

3.3.1.Molecularly imprinted polymer synthesis and characterization

3.3.1.1. Preparation of molecularly imprinted polymer

This research focused on determining the adsorption properties of MIP along with their physical and morphological characterization. Optimizing polymer synthesis with an efficient imprinting process plays a major role in molecular recognition properties (Karim et al., 2005). Non-covalent interactions including ionic, hydrophobic and hydrogen bonding between the template and the functional monomers (O'Mahony et al., 2005, 2006) drive the imprinting process with the aim of maintaining molecular recognition properties when the template is removed from the then "imprinted" polymers. Important synthesis parameters that influence the molecular recognition properties include the selection of monomers, polymerization technique (free radical, UV initiated) and polymerization parameters like temperature, duration of polymerization and porogen used.

Generally, creation of non-covalent interactions between the monomers and template followed by co-polymerization with cross linker using free radical polymerization (Ekberg and

Mosbach, 1989; Svenson et al., 2004) has been used for synthesis of MIP due to simplicity and production of high affinity and selective binding sites. High selectivity based on choice of monomers and crosslinkers (Yu and Mosbach, 1998, 2000) and also on pre-polymerization conditions (Hwang and Lee, 2002; Moring et al., 2002) has been well demonstrated. Spatial arrangements of shape selective cavities during the interactions between complementary functional groups of the template and the monomers is one of the driving forces behind selectivity of MIP (Spivak, 2005). ETA was used as a template molecule having stereochemistry representative of the ergot peptide family and because of the availability of well-established analytical methods for quantification (Amin and Sepp, 1976; Pierri et al., 1982; Krska et al., 2008a; Smith et al., 2009b; Jackson et al., 2012). The presence of a distinct ergoline structure and side chain tripeptide in ETA provides different functional groups capable of forming complementary interactions with functional monomers (e.g. styrene, HEMA). The ergoline portion of the ETA molecule includes one basic, tertiary amino group; one strongly polar heterocyclic, pyrrole ring; one amide group; and several low polarity hydrocarbon fragments (e.g., benzene ring, double bond) that can interact with monomers and crosslinker. The hydroxyl group of HEMA simultaneously functions as a hydrogen donor as well as a hydrogen acceptor, whereas styrene acting as an electron rich π donor could complement the π deficient ergot alkaloid rings, via a π- π stacking interaction. Studies have shown weak π-π stacking interactions between aromatic groups involving phenol and ethynylbenzene (Vojta and Vazdar, 2014) and aromatic rings of biomolecules (Waters, 2002). Additionally, the carbonyl and alcohol groups in ETA enable a range of non-covalent electrostatic interactions during pre-polymerization that are conducive to the formation of highly porous polymers after removal of the template.

3.3.1.2. Template removal

An important step in the process of creating imprints with high selectivity and absorption capacity is the removal of the imprinted template, especially because the imprint cavities of interest, i.e. those with the highest binding affinity, will most strongly retain the template molecules during template washing. For low molecular weight template molecules, highly crosslinked polymers are used to ensure preservation of the imprint cavity after removal of the template. However, for large template molecules, high crosslink densities seriously hinder mass transfer of the template, leading to slow template removal and therefore affecting its rebinding kinetics (Kulikova et al., 2010; Li et al., 2014). In the worst case, permanent

entrapment of the template in the polymer network could occur due to strong chemical interactions (Valdebenito et al., 2010), requiring an alternative imprinting approach like suspension polymerization.

The non-covalent interactions are generally attenuated using mild acidic or basic organic solvents which facilitate removal of template from the polymerized network. Several washing methods have been developed and optimized for template extraction (Ellwanger et al., 2001; Yang et al., 2004a) when electrostatic force, hydrophobic interaction or hydrogen bonding are involved, especially with large biomolecular templates (e.g., lysozyme, cytochrome; Hjerten et al., 1997; Chen et al., 2008). In our study, even though the template (ETA) was not as bulky as protein molecules, it is a large biomolecule (MW = 581.673 $g \cdot mol^{-1}$) with several functional groups (carbonyl, hydroxyl, amide and benzyl) that can possibly interact via ionic, hydrophobic, π-π, and electrostatic interactions, along with hydrogen bonding. Such interactions could lead to slow template release, and also to immobilization in the polymer matrix.

In the present study, polymers were washed (10x) in 0.2N HCl in methanol followed by 0.01M sodium citrate buffer of pH 4 and DI water wash over three days to remove the template. Additional washings were performed in methanol (5x) and acetonitrile (5x) followed by DI water until the last wash fraction contained negligible amount of ETA. The washings accounted for a total removal of 96% of the bound ETA from the polymer matrix (1.42g out of 1.81 g ETA bound to polymer). A considerable amount of template bound in the polymer (4%) is suggestive of the immobilization of ETA in the polymer matrix either due to strong interaction between the template and polymer, or to permanent entrapment of ETA in the polymer. Therefore, a sample of imprinted polymer (0.5g) was mixed in methanol and incubated for 14 days to confirm bleeding of ETA (data not shown). Analyzing the extracts on d 7 and d 14 showed very minor quantities of ETA release which accounted for a total of 0.0144 g out of 0.39 g that was strongly bound in polymer. This indicates that nearly 4% of the initial amount of ETA added was irreversibly bound to the polymer (i.e., nearly 4.13 $\mu g \cdot mg^{-1}$ of polymer) which may affect the specificity of the imprinted polymer. Studies have shown that harsh methods do not guarantee complete template removal (Ellwanger et al., 2001; Hawkins et al., 2005; Bonini et al., 2007), and such harsh treatments may ultimately affect the network structure of polymers, causing loss of affinity and/or specificity in rebinding (Hawkins et al., 2005).

Additionally, studies have shown the effect of molecular weight of template on imprinting. Although molecular imprinting has been particularly effective for molecules with low molecular weight (<1500 Da) (Flavin and Resmini, 2009; Huang et al., 2009; Verheyen et al., 2011; Li et al., 2014), reports of imprinting with larger biomolecular templates have increased considerably during the past two decades (Spivak and Shea, 2001; Bolisay et al., 2006; Seidler et al., 2009). However, selective recognition of large molecules appears to be extremely challenging due to fundamental difficulties with respect to monomer selection, washing method/template removal, quantification of rebinding and reproducibility (Turner et al., 2006; Takeuchi and Hishiya, 2008).

The difficulty in releasing the template from an MIP is a recognized drawback of the approach, especially when used as solid phase extraction material (Ellwanger et al., 2001). The phenomenon known as “bleeding” of the MIP is a remnant release of template in small quantities coming from leakage of physically entrapped template facilitated by swelling and shrinkage of the polymer material, or formation of template clusters that could be released by the polymeric material (Sellergren and Hall, 2001). The recovery of ETA following the initial ten acidic methanol washes was low (Figure 3-2). Among the ten washes, the sixth and the tenth wash had higher recoveries due to the overnight extraction periods. Additionally, after several washes using methanol and acetonitrile, total recovery of template was not possible. A total recovery of 67-88% was reported when copolymers of methacrylic acid and 2-hydroxyethyl methacrylate were imprinted with theophylline and extracted with acidic methanol (Tunc et al., 2006). Techniques like thermal annealing, microwave assisted extraction, Soxhlet extraction and super critical fluid template desorption, (Ellwanger et al., 2001; Peng-Ju et al., 2007) have also led to incomplete template recovery.

Quantitatively, most template removal occurred following five methanol washes. The efficiency of methanol to extract template mainly depends on its ability to penetrate the polymer and break the non-covalent bonds between the template and the polymer in a time-dependent fashion. Template release is also controlled by the integrity of the polymer which is dependent on the composition of functional monomers (Sellergren, 1999). In the present study, the monomer to crosslinker ratio was 3:10 on a molar basis, which was based on selectivity criteria observed between three different ratios of monomer:crosslinker (3:2.5, 3:5 and 3:10) tested in a preliminary study (data not shown) and also observed in other studies screening

different ratios of aromatic template and functional monomers (Nantasenamat et al., 2006). Additionally, MIP synthesized using methacrylate and EGDMA as monomer and crosslinker in a ratio of 1:4, respectively, exhibited better binding properties and selectivity towards ergot alkaloids, when metergoline was used as template (Lenain et al., 2012). Overall, 1.81 g of ETA was retained in the 91.92 grams of MIP pre-polymerization complex and approximately 81% (1.46g) was recovered during the template removal process.

3.3.1.3. Morphological characterization

3.3.1.3.1. Nitrogen sorption porosimetry

Both polymers exhibited responses typical of mesoporous particles (Figure 3-3). The increase in volume of N_2 adsorbed with the increase in relative pressure could be attributed to the capillary condensation of nitrogen into pores of polymer. The specific surface area and pore volume were higher for MIP compared to NIP (Table 3-1). A significant proportion of mesopores (5-8 nm pore size) was evident in both polymers with a cumulative pore volume of 1.1 and 0.613 cm^3/g for MIP and NIP, respectively. The comparatively large BET surface area and pore volume of MIP (431 m^2g^{-1}, 0.9-1.1 cm^3g^{-1}, respectively) were presumably created when the imprinting template molecule was removed from the polymer.

3.3.1.3.2. Microscopy

The SEM and TEM micrographs of MIP and NIP in their dry form revealed non-uniform dispersions of particles with irregular morphologies (Figure 3-4). NIP had a more regular structure than MIP. The surface of MIP exhibited more cavities than NIP, probably caused by imprinting of ETA, suggesting changes in structural properties of MIP due to the presence of template, which is expected. The morphology of polymers evaluated by TEM (Figure 3-4, C and D) suggested that both polymer particles were amorphous in nature. Free radical polymerization with concentrated solutions of monomers and high levels of crosslinkers typically yield amorphous polymers. The amorphous nature of the imprinted and non-imprinted polymers synthesized using free radical polymerizations was also observed in other studies using X-ray diffraction (Lanza et al., 2002; Turner et al., 2010). Such polymers have been shown to have numerous non-uniform distributions of binding sites producing non-linear binding isotherms and lower saturation capacities (Sellergren and Shea, 1993; Umpleby et al., 2001b).

3.3.1.3.3. Dynamic Light Scattering

The range of particle size distribution is a function of monomer and polymer conversion, chain length propagation and rate of chain length termination during polymerization as described by the power law (Matyjaszewski and Davis, 2002). Additionally, with bulk polymerization, mechanical grinding of polymer along with the swelling properties in different solvents may contribute to the wide range of particle size distribution. Figure 3-5 shows the hydrodynamic diameters of MIP and NIP determined by DLS. Tri-modal distributions of particle size were observed for both polymers in water and in 5% aqueous methanol. The particle size distribution had a broader range in 5% methanol compared to media containing DI water. Irrespective of the media, most particles were in the range of 5 to 90 µm with a peak in the range of 38-57 µm. In DI water, slight aggregation was noticed indicating inter-particulate hydrophobic interactions between the polymer molecules yielding a non-distinct continuous particle size distribution. This observation indicated that polymer sizes dispersed in adsorption media were similar between MIP and NIP.

3.3.1.4. Adsorption studies

3.3.1.4.1. Isothermal adsorption study

Langmuir adsorption isotherms quantitatively describe homogeneous monolayer adsorption between solids and liquids and assume identical adsorption sites of uniform energies. The model holds good when there is a finite number of adsorption sites on the outer surface of adsorbent and no transmigration of adsorbate on the plane of the surface (Langmuir, 1918; Vermeulan et al., 1966; Belton, 1976). On the other hand, Freundlich isotherms describe heterogeneous surface adsorption on the adsorbent with multiple layers of adsorption (Hutson and Yang, 1997). Langmuir and Freundlich constants derived from isothermal adsorption are presented in table 3-2. Figures 3-6 and 3-7 are Langmuir and Freundlich plots, respectively, for the adsorption of ETA onto polymers. In both cases, good correlation coefficients (R^2> 0.9) were obtained, which reveal the applicability of these isotherms. Studies have shown that adsorbents with heterogeneous binding sites usually show good fit with non-linear models like Langmuir and Freundlich (Umpleby et al., 2001b; Okeola and Odebunmi, 2010). For both models, residuals were randomly scattered around zero indicating that both reasonably described the data and normal probability plots suggested that the random errors affecting the adsorption process were normally distributed.

Even though both models had good fit, the Langmuir model provided a better fit than the Freundlich model, with R^2 greater than 0.95. The AICc, a measure that is used for comparing validity within a cohort of nonlinear models and frequently used for model selection (Burnham and Anderson, 2003; Spiess and Neumeyer, 2010), indicated a better fit with Langmuir (88 – 97% probability of being correct) compared to Freundlich model (3 -12% probability of being correct) for both polymers. Additionally, for both polymers, the absolute sum of squares was lower with Langmuir (9.18 and 8.62 for MIP and NIP, respectively) compared with Freundlich model (10.68 and 11.12 for MIP and NIP, respectively). Thus, results suggest that ergot alkaloid molecules bind to the surface of the polymer with low propensity to dissociate from that surface. The model selection also indicates that alkaloids are bound in a single layer to essentially equivalent sites (homogeneous) on the surface of the solid.

The important features of Langmuir isotherms are the adsorption constants, K_L, R_L and Q_o (Table 3-2). The K_L refer to binding site affinity, a factor that relates to heat of adsorption (Weber and Chakravorti, 1974). The affinity of adsorption depends on the activity coefficient of occupied and unoccupied sites on the adsorbents at equilibrium (Graham, 1953) which relates to the molar concentrations of adsorbate in the media. A positive K_L indicates that the interactions are spontaneous and energetically favorable (Sun et al., 2013), which was noticed for both polymers (K_L >0.37) in our study.

Affinity is a parameter that describes the binding of substances in equilibrium. Polymer geometry, polymer hydration, crosslink density, template size and temperature all play an important role in the time needed for large molecular templates to diffuse into the polymer matrix to reach equilibrium (Clapper and Guymon, 2007; Liechty et al., 2010). A substantial part of the literature investigating rebinding studies confirm an imprinting effect but lack convincing data on reaching the equilibrium state, especially with large molecule templates. Even though some studies have adjusted the incubation period to account for equilibrium in rebinding studies (Kimhi and Bianco-Peled, 2002, 2007), most researchers have used shorter incubation periods without reaching equilibrium, either to compare different polymers for adsorption properties, or to match with the limits of application of the finished polymer products (Schirmer and Meisel, 2006; Oxelbark et al., 2007; Pichon and Chapuis-Hugon, 2008; Janiak et al., 2009). Even though adsorption desorption was noticed up to 14 days (noticed while washing of MIPs to

determine template bleeding), all our isothermal adsorption studies were conducted for 90 minutes, beyond which there was minimal change in equilibrium.

Another parameter that effect the affinity is the medium type that influence the type of interaction. The strength of interaction between the imprinted polymer and analyte/template would also vary greatly depending on the properties of solvents used (Zhang et al., 2015). Hydrogen bonding interactions greatly contribute to the affinity of MIPs for low molecular weight compounds especially in organic or aprotic solvents and these interactions are generally hampered in aqueous media. In contrast to the few strong bonds that are responsible for the selective interaction between small molecular template and polymer in aprotic organic solvents, multiple weak interactions between the large molecules and the polymer network are necessary for the generation of a strong binding in aqueous environment (Hjerten et al., 1997; Sellergren and Hall, 2001). Electrostatic interactions seem to play a primary role in recognition if the selectivity is not altered by varying the water concentration of the binding media. On the contrary, in case of hydrogen bonding, the interaction between the polymer and template can be suppressed by increasing the concentration of the compound that has higher hydrogen bonding capacity (e.g., methanol or water) in the binding media. In addition, it is well known that the diffusion kinetics of large template in a highly crosslinked polymer matrix is a function of its molecular weight, with the slow diffusion coefficients for large molecules (Chen et al., 2011; Verheyen et al., 2011). All our rebinding studies were conducted in aqueous buffer media, thus suggesting that the interaction between ergolines and polymers may have occurred predominantly by hydrophobic binding, which could help explain the lack of substantial differences in binding between MIP and NIP.

Another constant that is calculated from Langmuir constant (K_L) is the separation factor R_L. The constant R_L is a dimensionless constant that indicates favorability of adsorption (Nwabanne and Igbokwe, 2008). The equation to determine the R_L value is shown in Table 3-2. An R_L value between 0 and 1 signifies favorable adsorption and R_L>1, R_L=1 and R_L=0 indicate unfavorable, linear and irreversible adsorption, respectively (Foo and Hameed, 2010; Sun et al., 2013). The R_L value in the present study was found to be between 0.37 and 0.38 for both polymers indicating adsorption between ETA and polymer was favorable. However, there was no difference between the polymer types.

The Langmuir constant Q_o in the Langmuir isotherm represents the practical limiting adsorption capacity and is useful in comparing performance of different adsorbents. It correlates well with specific surface area (Dunicz, 1961) and it relates directly with the amount of adsorbate bound from the solution. Results showed that there was no difference in the maximum adsorption capacity (Q_o) between the polymers, although Q_o was numerically greater for MIP (8.68 $\mu mol \cdot g^{-1}$) than for NIP (7.55 $\mu mol \cdot g^{-1}$) in pH 6.8, 0.1 M phosphate buffer at 39 °C, which could be due to larger surface area of the MIP created by the imprinting process. Nitrogen adsorption/desorption isotherms (BET) showed that the surface area of MIP was approximately twice that of NIP. However, surface area differences did not appear to affect the adsorption properties of MIP.

3.3.1.4.2. Selectivity study

Adsorption isotherms in the presence of structurally related alkaloids were used to evaluate selectivity of MIP vs NIP. Ergotamine tartrate has a weak acidic hydroxyl group and an amide moiety that provide unique functional groups necessary for specific interactions with polymer during imprinting. In addition, π-π interactions between the tetracyclic ring structure of ETA and styrene may enhance the interaction between template and polymer. The effect of imprinting on selectivity of different ergot alkaloids is shown in Figure 3-8. The adsorption coefficient, selectivity coefficient and imprinting selectivity data for adsorption selectivity of polymers towards ergot alkaloids are shown in Table 3-3.

Both polymers exhibited similar adsorption coefficients (k) to ETA and BC, but had lower affinities towards ME and LY. There was significant interaction (P <0.001) between the product and the alkaloid types with respect to adsorption coefficients. Although there was comparatively greater adsorption of ME and LY for MIP compared with NIP (as indicated by k' values of 25 and 50), adsorption of both compounds was relatively low compared to that for ETA and BC for both products. There was no difference (P =0.24) between the polymers when alkaloids were grouped as ergopeptines and ergolines (ergopeptines: ETA and BC; ergolines: ME and LY). In reference to selectivity coefficient, (k'), higher k' value indicates better selectivity of polymer towards rebinding of template (ETA). Comparing k' values between polymers, it was evident that both MIP and NIP selectively bound ETA in the presence of ME and LY, however, NIP exhibited greater selectivity (k') compared to MIP. The k'' is intended to evaluate the imprinting effect, where higher coefficients indicate greater selectivity towards the template.

Additionally, there was minimal imprinting effect that was indicated by values of *K''* that is ≤ 1 (Table 3). The k'' values were slightly above 1 for BC, these ratios were the consequence of small differences between MIP and NIP at comparatively low levels of alkaloid binding. Similar results were obtained for selectivities of phenolic compounds, where minimal differences between MIP and NIP were noticed when hydrophobic interactions were involved [100]. This suggested that interaction with alkaloids on the product surface via hydrophobic interaction or π-stacking were common between the MIP and NIP, thereby yielding minimal difference between products.

3.3.1.4.3. Cross-reactivity experiment

Cross reactivity was evaluated from the adsorption properties of MIP and NIP to frequently occurring mycotoxins that can be found in similar sample matrices in similar concentration ranges as the investigated ergot alkaloids. Different mycotoxins with different functional groups (carboxylic acid, and amines), pKa's (acidic, neutral and basic) and solubilities were selected. Both polymers exhibited non-specific adsorption to different toxins (Figure 3-9A) to different degrees and there was no difference in mean adsorption between MIP and NIP for any of the mycotoxins. Toxins were grouped according to their structural conformation and functional groups (Figure 3-9C). The group of toxins with low solubility in water and having basic pKa's are either neutral or positively charged in the adsorption medium of pH 6.8. These molecules (ZEA, Roc Q and STG) are polycyclic, have the possibility of exhibiting hydrophobic interactions, and showed more than 80% adsorption. A second group of toxins included molecules that are more hydrophilic in nature. These molecules (DON, FA and OTA) have low pKa values and exhibited a low degree of interaction with polymers. The last group of toxins included polycyclic compounds (AFB1, CPA and DAS) which have a tendency to dissolve in water. This group of toxins demonstrated an intermediate degree of adsorption to polymers which could be due to hydrophobic interactions. The adsorption was significantly different between the three groups of toxins ($P < 0.001$) (Figure 3-9B), however, there was no difference between the polymers in adsorbing any of these toxins ($P = 0.462$).

3.3.1.4.4. Effect of pH and temperature

The effect of pH on the adsorption of ETA to both polymers is shown in figure 3-10. The ETA adsorption ranged between 96 and 99 percent and there was no difference between the pH range ($P = 0.24$) or between the polymers ($P = 0.18$). The lack of effect of pH on the adsorption is

suggestive of non-ionic interactions, especially hydrophobic interactions as the primary driving force for adsorption.

Most of the contaminated feeds including fescue seeds and fescue grass or its products like haylage have a pH range between 3.5 and 8, and ruminal pH of animals grazing fescue would generally be between 6.2 and 6.8. Solution pH determines the degree of ionization of ETA and dictates its speciation in solution. Similarly, the pKa of the polymer in the adsorption media and the surface charge that can be influenced by solution pH have been shown to affect its adsorption properties (Carrott et al., 2008). Therefore, the ability of polymers to interact with ETA in different pH ranges is important. Under physiological conditions, the pKa of polymers made from acrylates including polymethylacrylic acid (Kim and Peppas, 2002) and poly-acrylic acid (Yang et al., 2004b) were shown to be nearly 5 and 4.28, respectively. In the present study, the adsorption efficiency remained similar at pH ranges between 2 and 10. Results indicate that pH effects would not limit the utility of the polymers under study as an extraction material for ergot alkaloids or as an adsorbent in the feed to reduce the bioavailability of ergot alkaloids.

Figure 3-11 shows the adsorption of ETA when 1 mg of polymer was exposed to 1 $\mu g \cdot mL^{-1}$ of ETA concentration in 0.01 M phosphate buffer (pH 6.8), at three different temperatures (36, 39 and 42 °C) that encompass the range of ± 3 °C around normal ruminal temperature of 39 °C. Even though the data for both polymers indicated that adsorption was reduced by nearly 10 percent when the temperature was 39 °C, the decrease was statistically insignificant ($P > 0.11$) and there was no difference ($P = 0.64$) between the polymer types at each temperature. Polymers have an innate tendency to swell or collapse with change in temperature causing changes in surface properties (Schmaljohann, 2006; Reinhardt et al., 2013). However, our studies indicated no difference in the adsorption properties of the polymer in between the temperature range of 36 to 42 °C. These data offer little evidence of specific binding by MIP. Other approaches such as evaluation of binding above and below glass transition temperatures could provide evidence for or against specific binding of MIP. However, those data are not currently available for these products.

3.3.1.4.5. Adsorption of ETA from rumen fluid solution

The adsorption efficiency of different levels of MIP to ETA in comparison to control (NIP) in complex media was determined. Rumen fluid is a complex medium that includes feed

residues, microbes, phenolic compounds, organic acids, soluble proteins, peptides, amino acids, etc. (Wang and McAllister, 2002). The adsorption efficiency of imprinted polymer to its template (ETA) in the presence of compounds that can interact via hydrophobic and π-stacking (phenolic compounds) may indicate its selectivity to the template. Comparative chromatograms from samples spiked with 3.3 $mg \cdot L^{-1}$ ETA in rumen fluid that were dosed with increasing levels of MIP are shown in Figure 3-12. With the increasing dose of polymer in rumen fluid, the peak area of ETA decreased. The maximum adsorption was noticed at 0.01% w/v (100 $mg \cdot L^{-1}$) of polymer with maximum efficiency of 97 percent for MIP and 85 percent for NIP (Figure 3-13). There was minimal increase in adsorption with higher concentrations of polymer indicating the optimal dosage of polymer to adsorb 3.3 $mg.L^{-1}$ of ETA from rumen fluid was 0.01% w/v (100 $mg \cdot L^{-1}$).

Adsorption efficiency to ETA with dose titration of polymer provides information for inclusion rate of polymer for its application as an adsorbent when used as a feed supplement for mitigation of ergot toxicity. By using ruminal fluid (pH 6.8) as media, a representation of adsorption efficacy in complex media was evaluated. The selectivity of the polymer towards the template (ETA) was tested to determine the effective dose of polymer required to reduce the concentration of ETA by at least 90 percent. Generally, the concentration of ergovaline in naturally contaminated tall fescue seed and forage range between 300 and 7000 $\mu g \cdot kg^{-1}$ of dry matter (Rottinghaus et al., 1991; Krska and Crews, 2008). In the present study, the calculated mean ETA concentration in the ruminal fluid was 3.3 $\mu g \cdot mL^{-1}$, which is twice higher order of magnitude compared to ergovaline concentrations that would be normally present in rumen fluid of animal grazing endophyte infected tall fescue pasture. Since both ergovaline and ETA are ergopeptides alkaloids that have similar ergoline structure and pharmacodynamics properties (Porter, 1995; Larson et al., 1999), ETA was used as the reference alkaloid in the present binding studies. At inclusion rate 0.01% w/v (100 $mg \cdot L^{-1}$) and above, the average adsorption efficiencies for MIP was greater than 97%. However, at similar inclusion rates NIP exhibited significantly ($p<0.01$) lower adsorption. There was no interaction ($P= 0.23$) between inclusion rate and polymer type and the mean adsorption of the NIP was almost ten percentage points lower than that of MIP. This phenomenon may be attributed to the larger surface area available for adsorption in MIPs compared to NIP.

3.3.1.5. Fourier Transformed Infrared (FTIR)

Figure 3-14 illustrates the FTIR spectra of monomer solutions (styrene, EGDMA, HEMA) and their MIP. Overlaid spectra comparing MIP and NIP and also MIP and [MIP + ETA] complex over the frequency range of 4000-650 cm^{-1} are shown in Figure 3-15 and 16, respectively. Absorption peaks for C=C, typical for the ring structure of styrene were observed at 1600, 1494 and 1449 cm^{-1} and the presence of sp2 hybridization in the styrene ring system was evident from the absorption spectrum below 3000 cm^{-1}. A broad absorption band for the hydroxyl group of HEMA was found at 3430 cm^{-1}. The C=O absorbance was evident at 1716 cm^{-1} in both HEMA and EGDMA. Upon polymerization, with free radical initiation, the involvement of functional groups including C=O, –OH and C=C were evident. Shifting or disappearance of peaks at different wave numbers between the monomers/crosslinkers and polymerized product demonstrate utilization of functional monomers in bonding during polymerization. Coordination through the hydroxyl or double bonds was evidenced by the disappearance of the peak for –OH of HEMA, Csp2-H hybrids of styrene, and C=C bonds of EGDMA. The ester bond is located at 1044 cm^{-1} before polymer formation and a shift to higher wave numbers indicates its minor role in either template recognition or polymer formation.

The FT-IR spectra of control MIP and NIP prepared by free radical polymerization are shown in Figure 3-15. Both polymers had similar IR spectra indicating the similarity in the functional groups and also suggesting maximum removal of ETA during template washing which is necessary to obtain clean imprinted polymer. The results also indicated minimum covalent bonds between the template and the polymer. The C=C band at 1729 cm^{-1} from NIP was of similar intensity compared to MIP, indicating similar backbone structure that is inherent in both polymer. Some of the characteristic bands of both polymers included -CH3 (asymmetry) at 2955 cm^{-1}, carbonyl stretch (-C=O) at 1724, -C-C- at 1635-1670 cm^{-1}, -CH3 (symmetry) at 1450 cm^{-1}, -C-O or –C-O-C- stretch at 1250 cm^{-1} and C-H vibrations at 756, 1388 cm^{-1}.

Figure 3-16 represents FTIR spectra of MIP before and after exposure to ETA in 0.1 M phosphate buffer of pH 6.8. The [MIP + ETA] complex was washed with DI water to remove free ETA and later freeze dried before analysis. The [MIP +ETA] complex showed all characteristic bands of the MIP polymer, however, there were changes in the bands at 2950 to 2970 cm^{-1} suggesting involvement of ring structures in adsorption via π-π stacking. There was no shift

occurring for the ester C=O suggesting an inconsequential effect of the ester in template recognition. These findings indicate that a significant degree of interaction between the template and the polymer is governed by π-π stacking. Additionally, the changes in the signal intensity of the CH_3 stretching frequency (2955 cm^{-1}) can be explained by a hydrophobic interaction of polymer compounds with neighboring template molecules in solution. The greater signal due to presence of water molecules surrounding the CH_3 groups may indicate contribution of hydrophobic effects in adsorption. The influence of water molecules on the hydrophobic effects has been shown and theoretically confirmed (Schmidt et al., 2005; Schmidt et al., 2006)

3.4. Conclusions

Styrene based imprinted polymers synthesized with ergotamine as template were tested for morphological and adsorption properties. MIP had almost twice the external surface area and pore volume compared to NIP, confirmed by the smaller pore size of MIP. The particle size distribution was tri-modal with particles of mean size of 50 microns representing nearly 10-15 percent of the population. Particle morphology determined using SEM and TEM suggested a highly porous nature of MIP. Exposure of polymers to high electron beam (TEM) for 5 min caused crystallization of amorphous polymers, suggesting distortion of soft particles or shrinkage.

The tetracyclic group of ETA was used as a representative molecule for structural recognition in the polymer. Results suggested that functional groups including indole NH, carbonyl and hydroxyl groups present on the template molecule can contribute to physical interaction with polymers via hydrogen bonding while the resonance property of ring structures could contribute to π-stacking interactions. Additionally, an alkylphenolic chain in the polymer provided more adsorptive surface area and may be responsible for hydrophobic interactions. Freundlich isotherms indicated that both polymers had favorable adsorption but no difference was noted between MIP and NIP. The difficulties in differentiating MIP and NIP could be due to similar surface functional groups that are normally present irrespective of imprinting. However, MIP had better adsorption efficiency towards ergotamine in rumen fluid compared to NIP, indicating some selectivity of adsorption in a complex *in situ* environment. Additionally, higher binding efficiency of MIP at low levels of ETA could be due to greater surface area. In addition,

ergopeptides exhibited better adsorption parameters compared to ergoline molecules indicating the importance of functional groups on the ergoline ring, including a tripeptide moiety.

The imprinted polymers have properties conducive to use as a sorbent in analytical extraction to remove ergot alkaloids from complex media like rumen fluid, which could find use in sample preparation prior to HPLC or LC-MS/MS analysis. Even though styrene based polymers have been utilized in a variety of studies for imprinting, many studies with biological applications have used methacrylate-based imprinted polymers. Imprinted polymers made from acrylate monomers have been evaluated as pH-responsive, slow drug delivery systems in the gut suggesting acceptability of polymethacrylates without adverse effects. Therefore, subsequent experiments were targeted towards synthesis of MAA-based biocompatible ergotamine-imprinted polymers with an ultimate goal of utilizing them as alkaloid adsorbents in animal feed to reduce bio-availability of ergot alkaloids in the gut.

Table 3-1. Surface area, pore volume and pore size of MIP and NIP using BET and BJH method.

	MIP	NIP
BET surface area, m^2/g	431.447	213.180
BJH cumulative pore volume cm^3/g		
Pores between 0.5 and 300 nm diameter	1.1157	0.613
Pores between 1 and 300 nm diameter	0.9552	0.545
BJH, average pore diameter, nm		
Adsorption	5.417	6.210
Desorption	7.776	7.578

Table 3-2. Langmuir and Freundlich isotherm adsorption parameters of ETA on to molecularly imprinted and non-imprinted polymers.

	Langmuir				Freundlich			
	Q_o	K_L	R_L	R^2	K_f	1/n	n	R^2
	($\mu mol \cdot g^{-1}$)	(L·g−1)			($\mu mol \cdot g^{-1}$)			
MIP	8.68 ± 0.80[a]	0.37 ± 0.14 [a]	0.38 [a]	0.95	2.48 ± 0.41 [a]	0.31 ± 0.04 [a]	3.16 [a]	0.94
NIP	7.55 ± 0.61 [a]	0.43 ± 0.01 [a]	0.37 [a]	0.96	2.40 ± 0.39 [a]	0.29 ± 0.04 [a]	3.37 [a]	0.92
Equation	$q_e = \frac{Q_o K_L C_e}{1 + K_L C_e}$		$R_L = \frac{1}{1 + K_L C_o}$		$q_e = K_f C_e^{\frac{1}{n}}$			

q_e: amount adsorbed, $\mu mol \cdot g^{-1}$; K_L: Langmuir adsorption constant; Q_o: maximum amount adsorbed ($\mu mol \bullet g^{-1}$); C_o: initial concentration of adsorbate ($\mu mol.L^{-1}$); C_e: is equilibrium concentration of adsorbate ($\mu mol.L^{-1}$); K_f: adsorption capacity factor and 1/n: adsorption intensity or surface heterogeneity index.

Data represent mean standard error of mean (n=3).

Values with different superscript within the column are significantly different (P <0.05)

Table 3-3. Adsorption coefficient (k), selectivity coefficient (k') and effect of imprinting (k'') of MIP and NIP for different ergot alkaloids.

Alkaloids	MIP		NIP		Imprinting effect
	k[1]	k'	k[1]	k'	k''
Ergotamine	0.924 ± 0.017[a]		0.910 ± 0.005[a]		
Methylergonovine	0.122± 0.006[a]	7.590 ± 0.403	0.037 ± 0.003[b]	24.97 ± 1.882	0.308 ± 0.025
2-Bromo-alpha-ergocryptine	0.755 ± 0.006[a]	1.223 ± 0.010	0.750 ± 0.011[a]	1.213 ± 0.018	1.009 ± 0.015
Lysergol	0.041± 0.003[a]	22.763 ± 0.192	0.018 ± 0.001[a]	50.063 ± 1.360	0.455 ± 0.012

Data represent the mean affinity value ± standard error of mean (n=3)

k : adsorption coefficient; k': selectivity coefficient; k'' : effect of imprinting on selectivity

[1]*k* values with different superscripts differ ($P < 0.01$) between MIP and NIP within a given alkaloid.

Polymer type x alkaloid type interacted ($P < 0.01$)

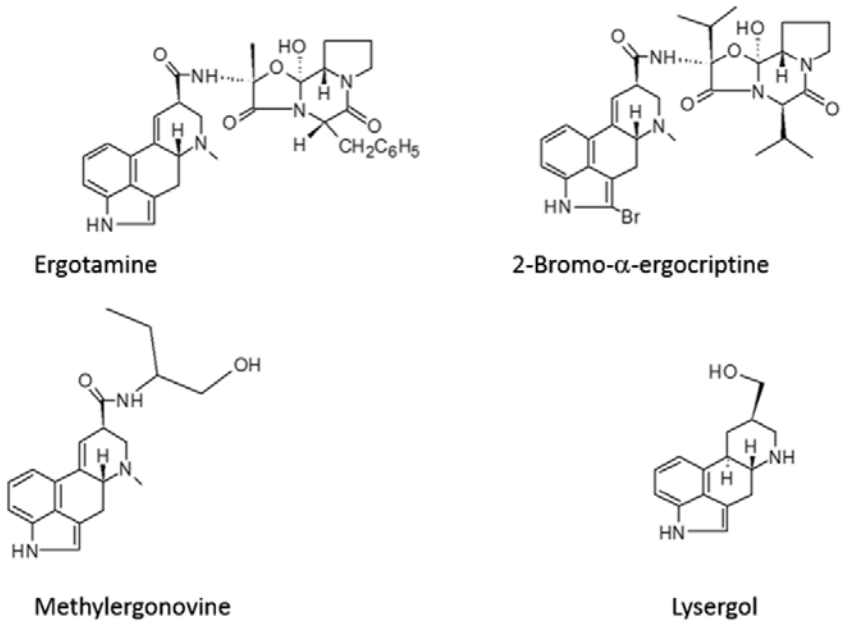


Figure 3-1.Molecular structures of different alkaloids used in selectivity experiment.

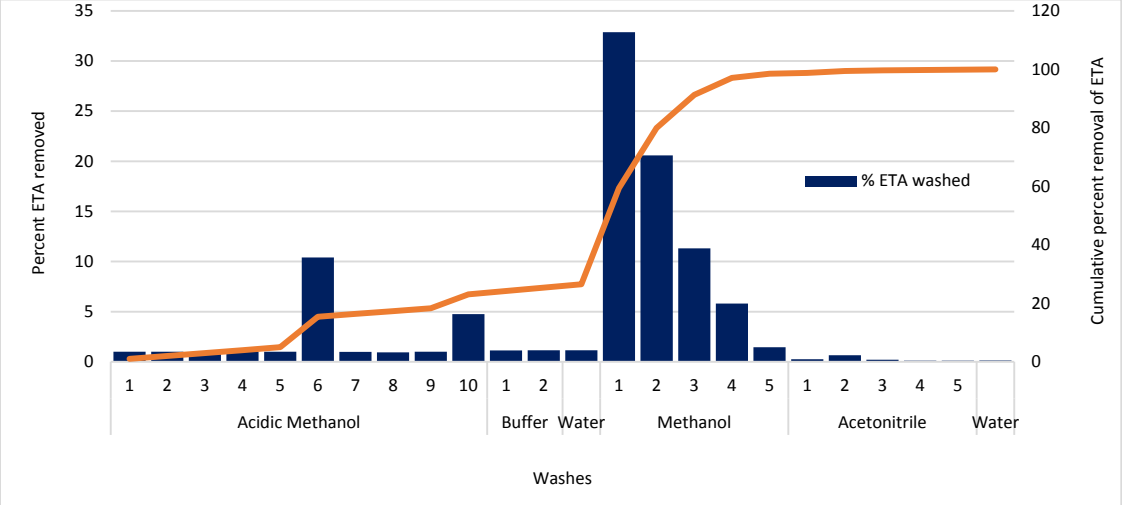


Figure 3-2. Removal of ETA from MIP during template wash procedure using different extraction solvents.

Acidic methanol: 0.2 N HCL in methanol
Buffer: 0.1M sodium citrate buffer of pH 4
Water: DI water

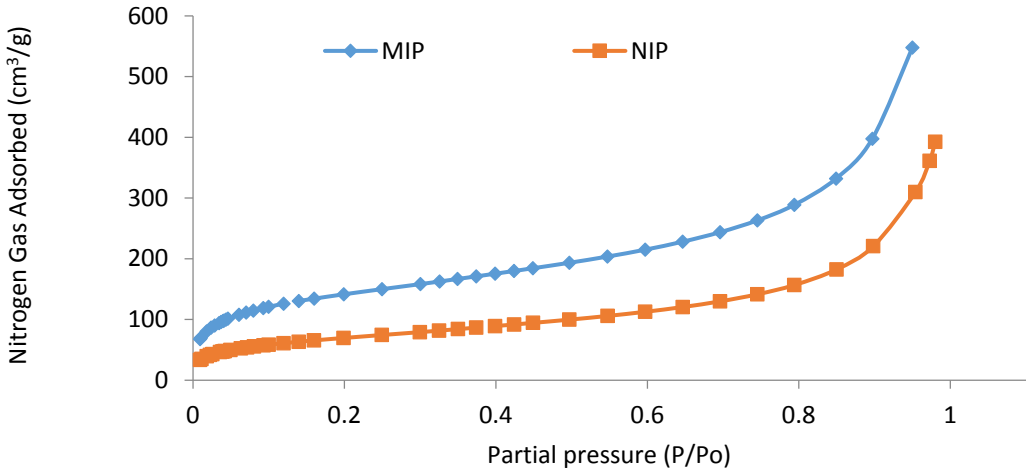


Figure 3-3. Adsorption isotherms in the mesoporous systems. Adsorption excess is given in units of cm3·g^{-1} adsorbate, plotted against relative pressure.

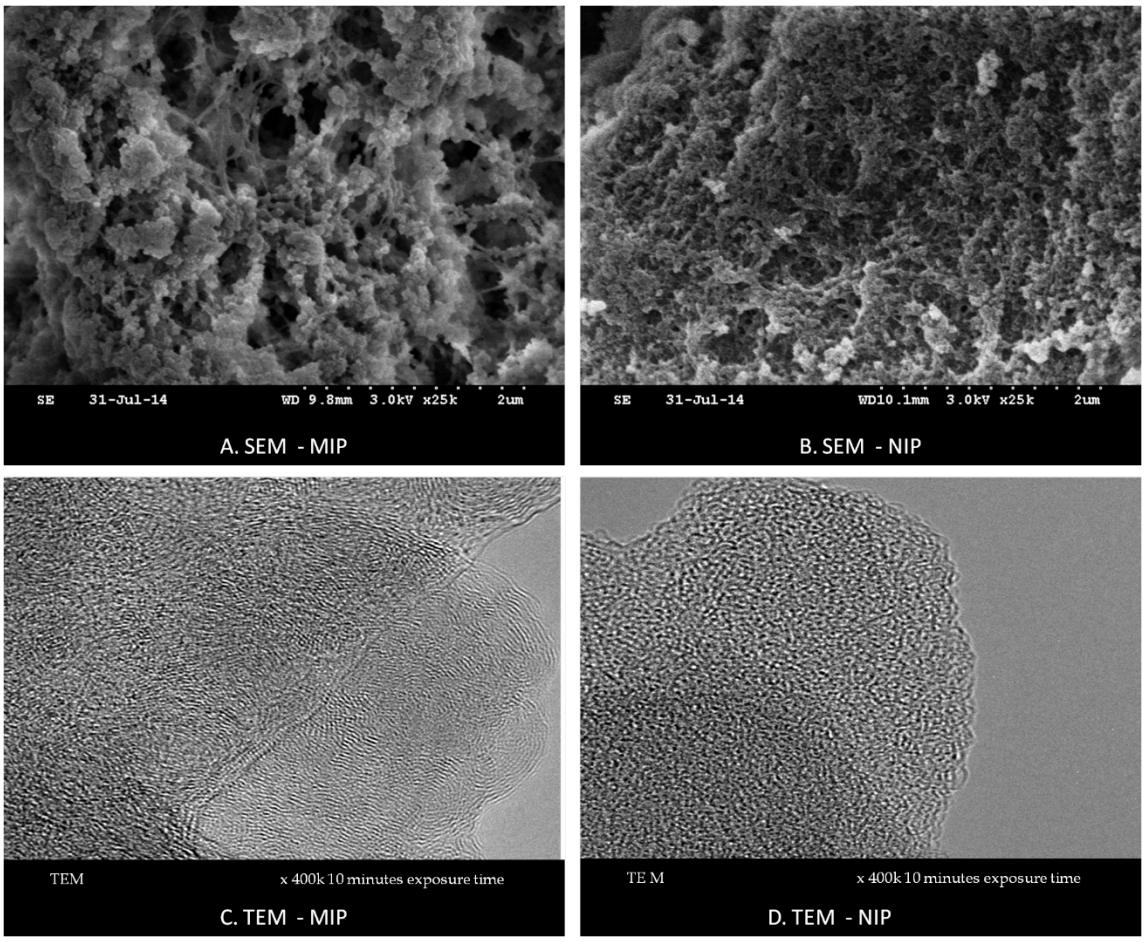


Figure 3-4. SEM and TEM images of polymers. A, B) SEM of MIP and NIP. C, D) TEM of MIP and NIP.

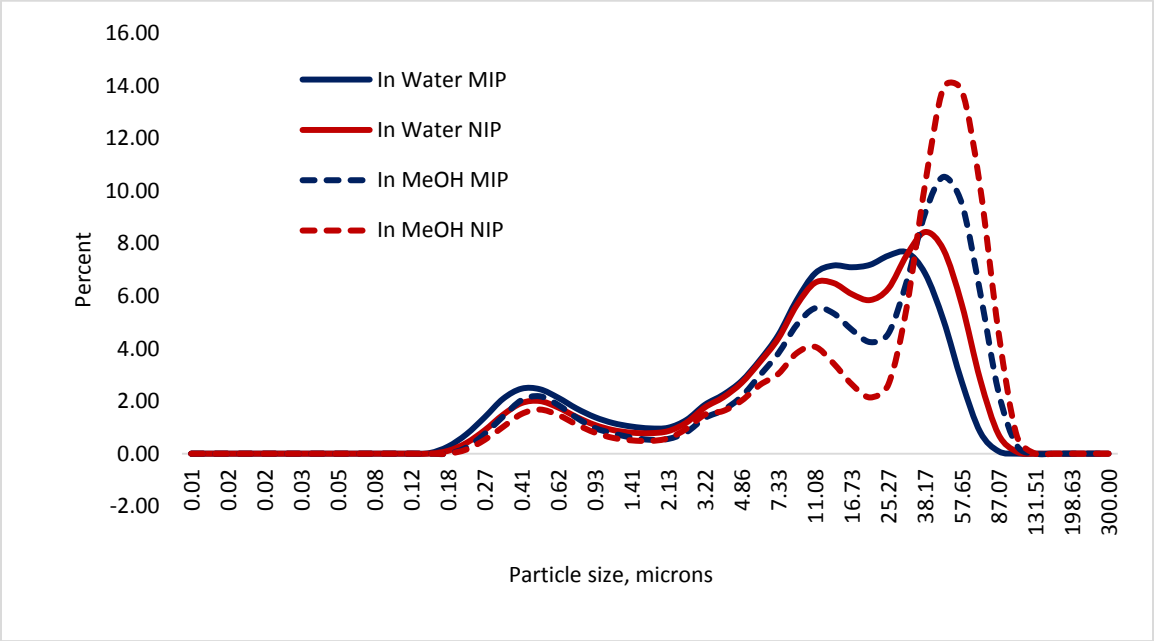


Figure 3-5. Hydrodynamic particle size distribution (%) of imprinted (blue) and non-imprinted (red) polymers in water (solid line) and in 5% methanol (dotted line) by dynamic light scattering (DLS) measurements.

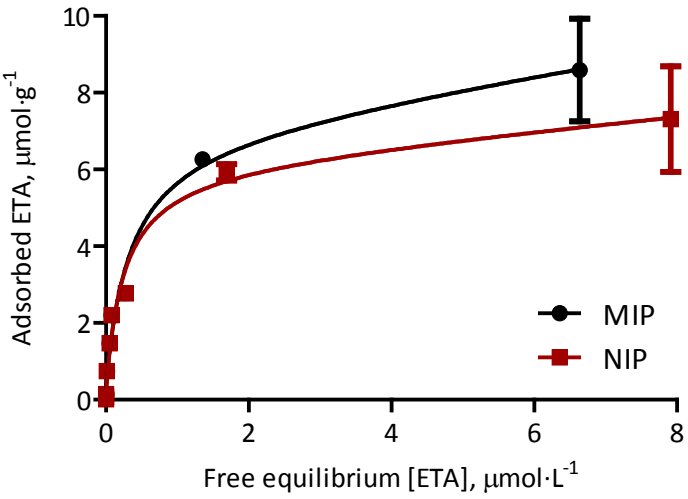


Figure 3-6. Langmuir isothermal adsorption plot of the bound concentration of ETA (µmol·g^{-1}) to MIP and NIP as a function of the concentration of free ETA at equilibrium. The data in the figure represents mean ± standard error of mean (n=3).

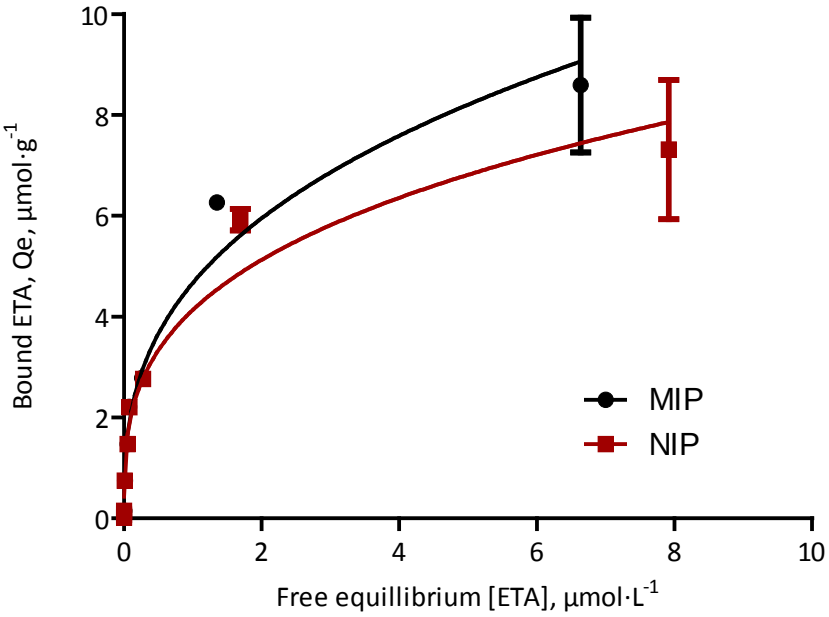


Figure 3-7. Freundlich isothermal adsorption plot of the bound concentration of ETA (µmol·g^{-1}) to MIP and NIP as a function of the concentration of free ETA at equilibrium. The data in the figure represents mean ± standard error of mean (n=3).

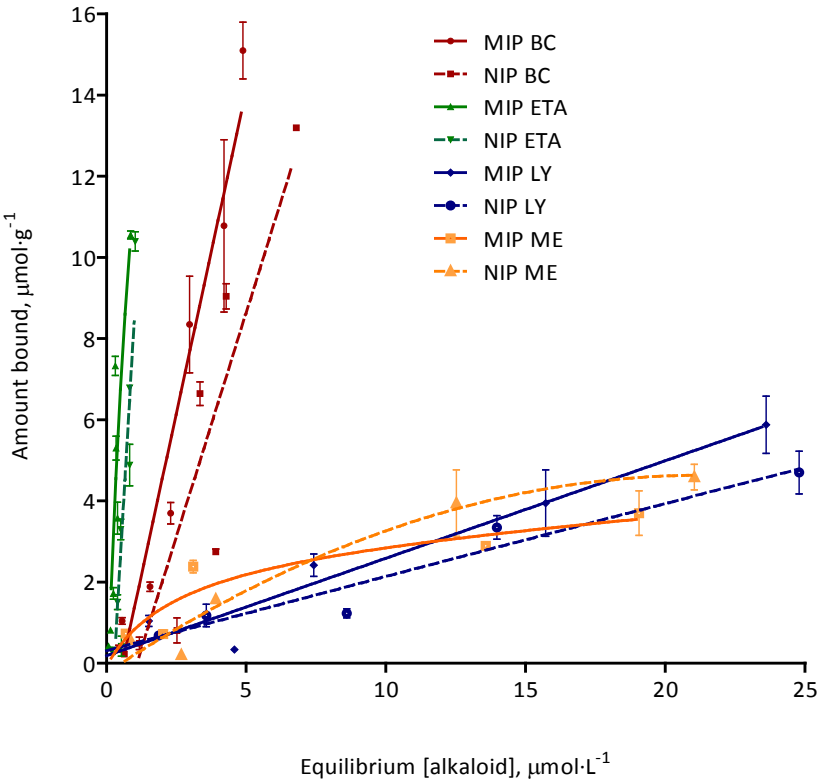


Figure 3-8. Isothermal competitive adsorption plot using one-site total binding model for ETA, 2-BC, ME and LY on imprinted (solid line) and non-imprinted (dotted line) polymer at inclusion rate of 0.1 mg·mL^{-1} in ammonium citrate buffer of pH 6.7. The data in the figure represents mean ± standard error of mean (n=3).

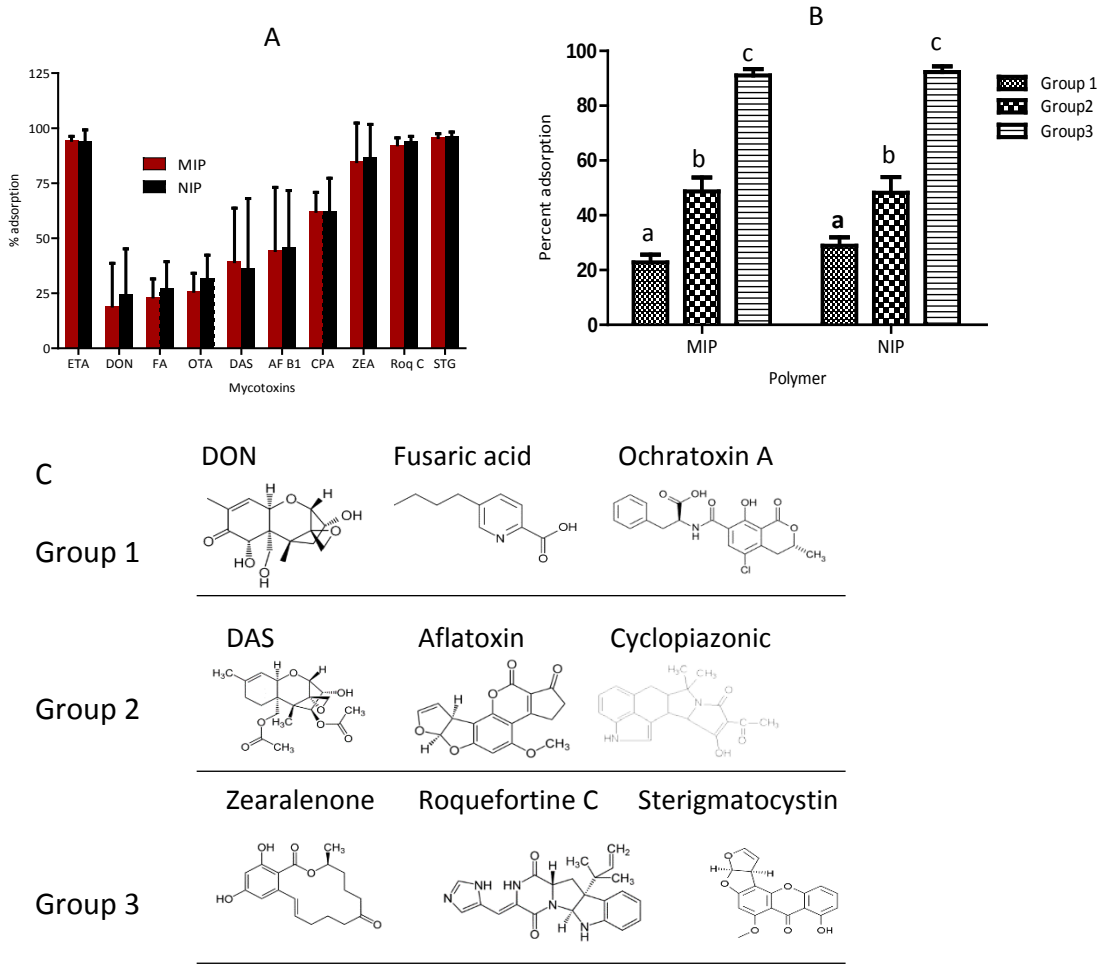


Figure 3-9. A) Cross reactivity of ten different mycotoxins with MIP or NIP (Mean ± SEM; n=3). B) Adsorption comparison of three groups of mycotoxins based on structure and pKa (Mean ± SEM; n=21); different letters indicate significant differences in adsorption treatment means (ANOVA: $P < 0.05$). C) Structure of different mycotoxins grouped based on structure and pKa. Adsorption study was conducted in 0.01M ammonium citrate buffer media of pH 6.7.

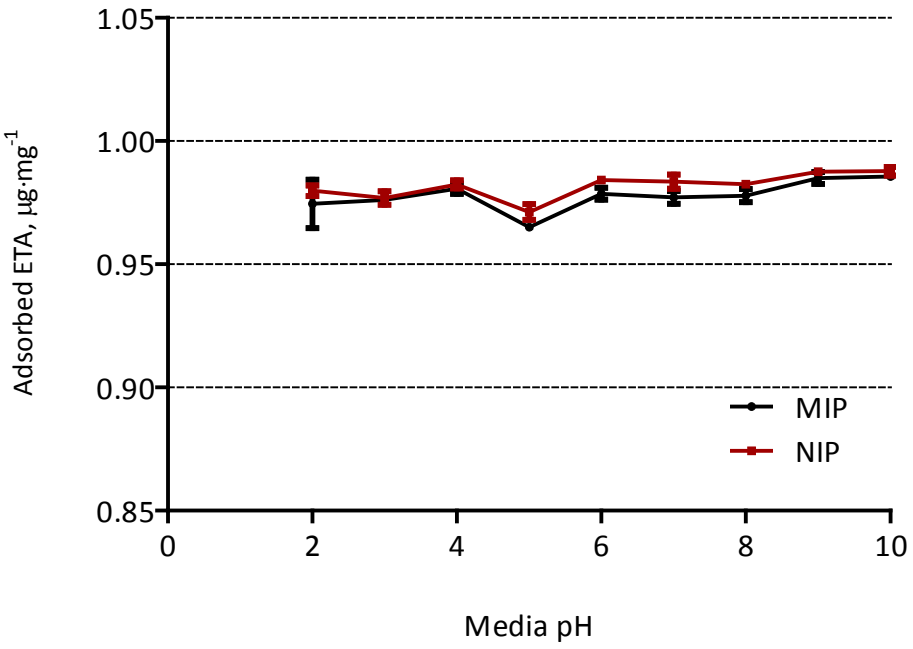


Figure 3-10. Effect of pH on the adsorption of ergotamine on MIP and NIP, conducted in 10 mL volume of phosphate buffer (0.1 M, pH 6.8), with 1 $mg \cdot L^{-1}$ initial ergotamine concentration and 1 mg of polymer dose. The data in the figure represents mean ± standard error of mean (n=3). There was no pH x polymer interaction (P = 0.64), no effect of pH on adsorption (P = 0.24) and no difference between MIP and NIP (P = 0.18).

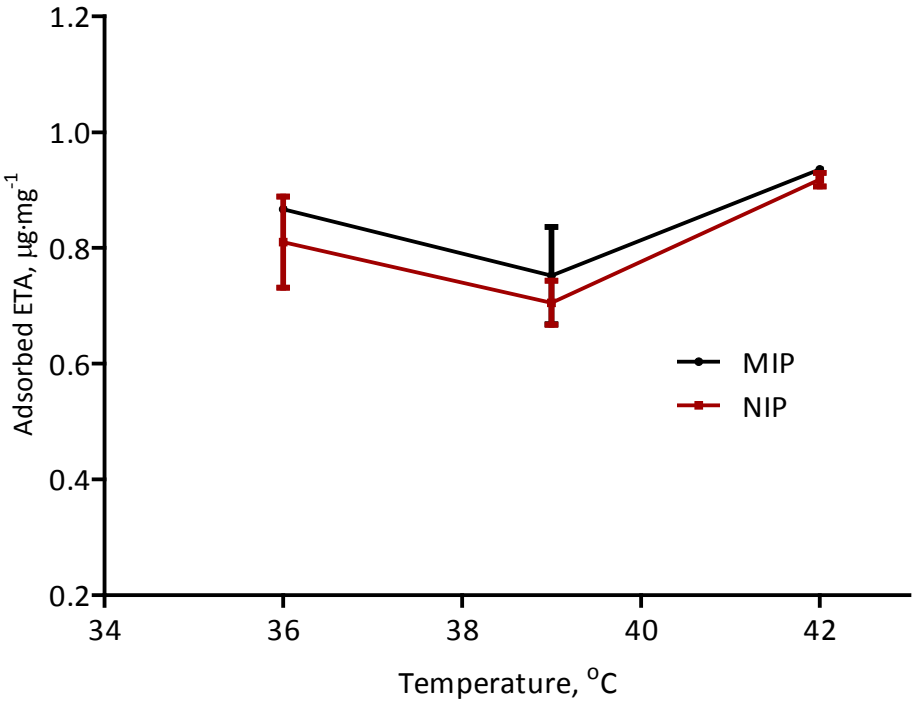


Figure 3-11. Effect of three temperatures on adsorption of ergotamine on MIP and NIP in phosphate buffer at pH 6.8 with inclusion of 1 mg of polymer and 1 mg·L^{-1} of ergotamine. The data in the figure represents mean ± standard error of mean (n=3). There was no interaction ($P = 0.78$) and no difference ($P > 0.11$) between the polymer type and between the mean temperatures.

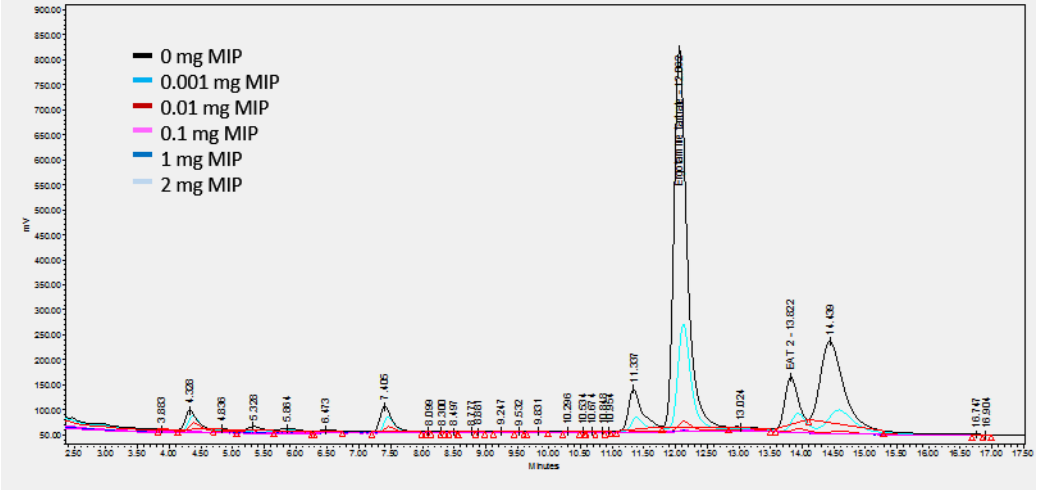


Figure 3-12. HPLC peaks of samples spiked with ergotamine (3.3 mg·L^{-1}) in rumen fluid dosed with five different levels of MIP (similar trend in peaks were obtained for NIP, except that the peak area was little larger than MIP).

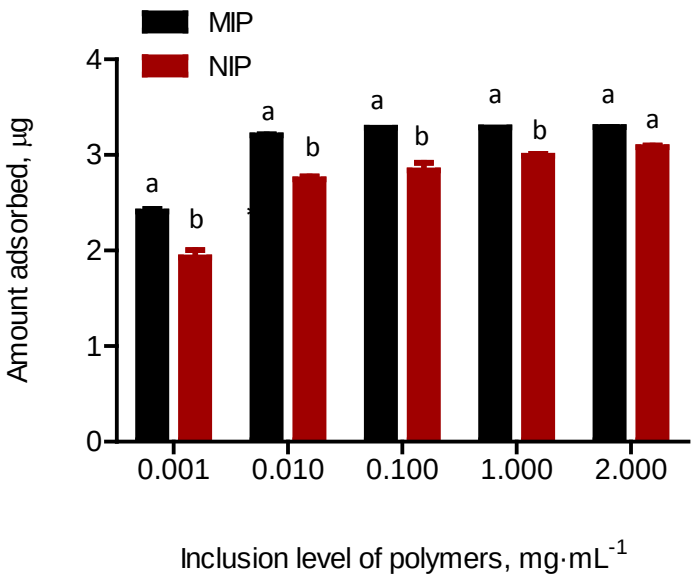


Figure 3-13. Adsorption efficiency of ergotamine (3.3 mg·L^{-1}) with increasing level of MIP and NIP in rumen fluid. The data in the figure represents mean ± standard error of mean (n=3). Means with different alphabets within the inclusion levels are significant different *(P* < 0.01).

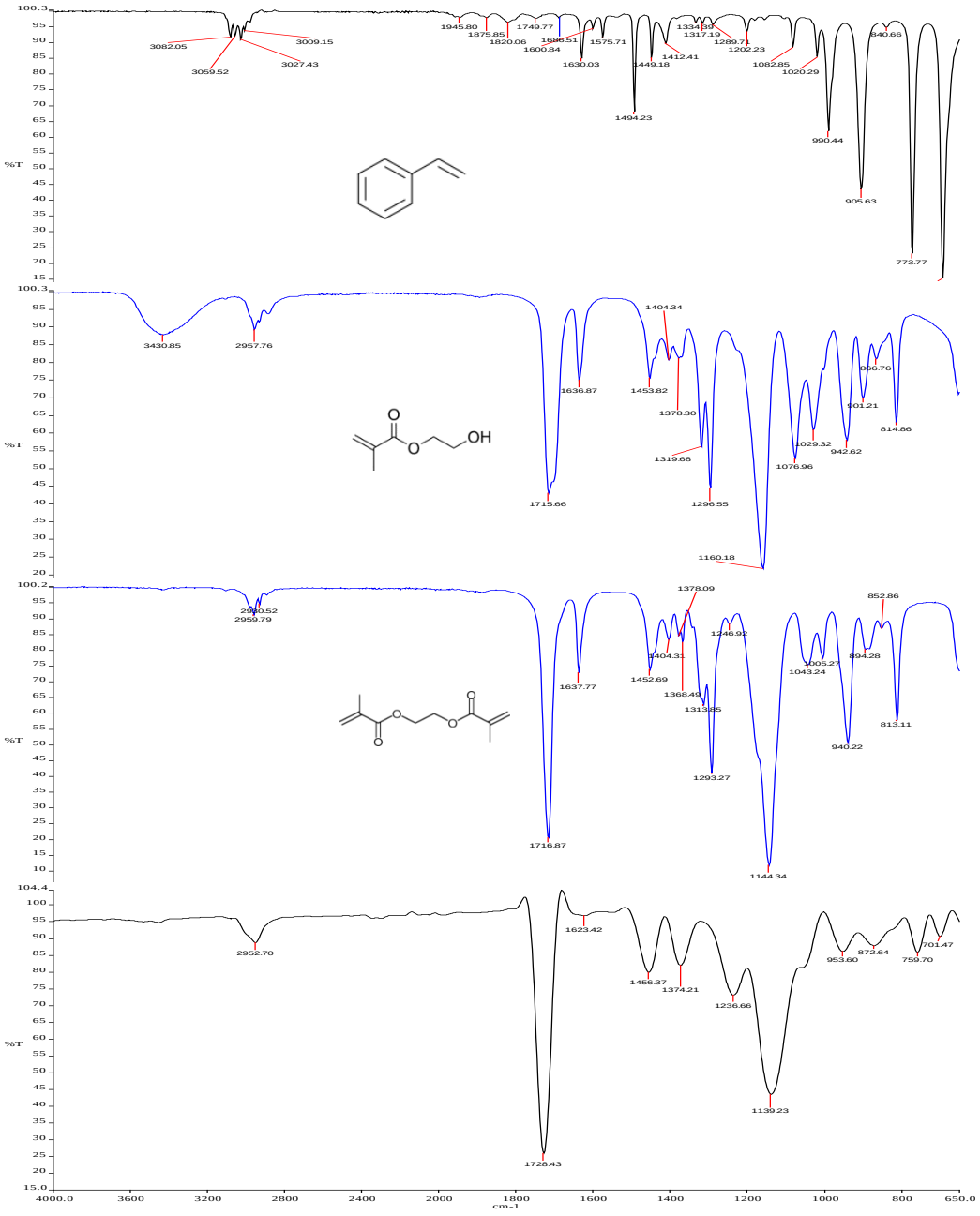


Figure 3-14. FTIR spectra of monomers and imprinted polymers recorded between the frequencies of 4000-650 cm^{-1}.

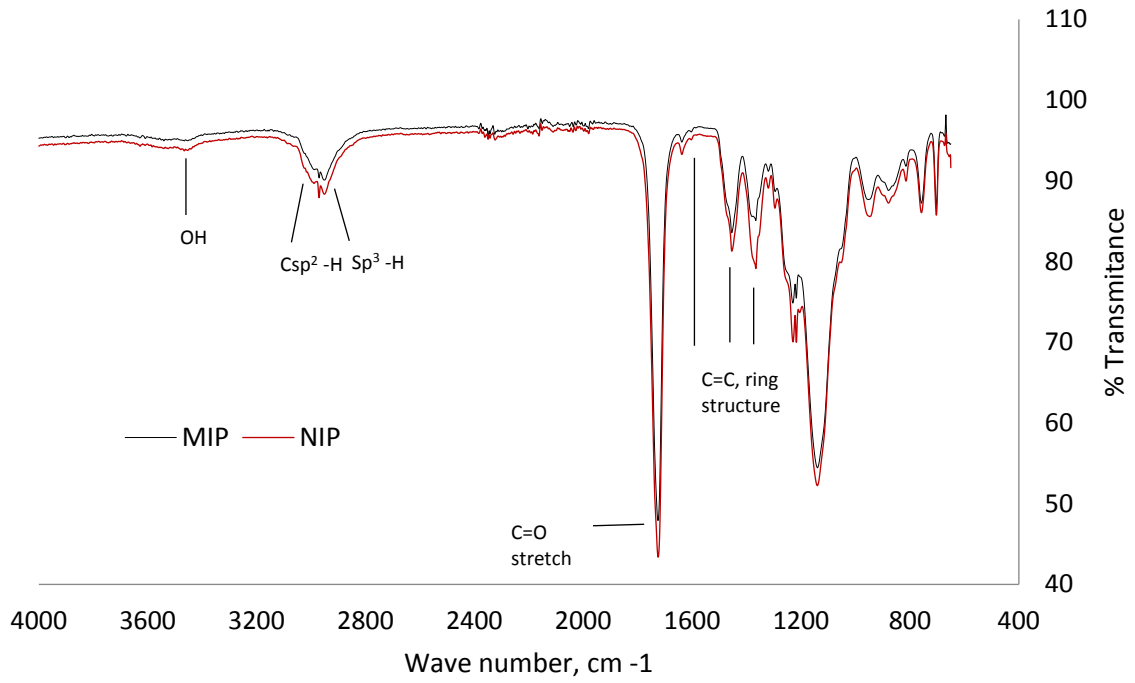


Figure 3-15. FTIR spectral comparison between the imprinted (MIP) and non-imprinted polymer (NIP) recorded between the frequency ranges of 4000-650 cm^{-1}.

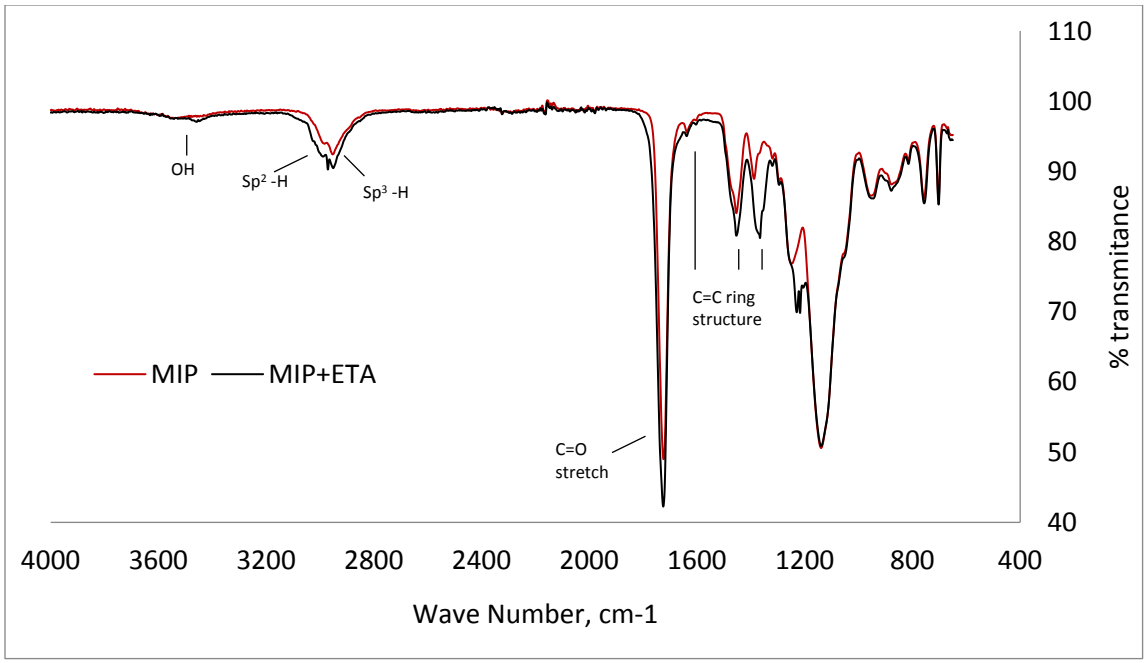


Figure 3-16. FTIR spectral comparison between the imprinted polymer (MIP) and ETA-MIP complex recorded between the frequency ranges of 4000-650 cm^{-1}.

4. CHARACTERIZATION OF HYDROXYETHYL METHACRYLIC ACID FUNCTIONALIZED METHACRYLATE BASED POLYMER IMPRINTED WITH ERGOTAMINE TEMPLATE FOR ERGOT ALKALOID ADSORPTION

4.1. Introduction

Animals consuming tall fescue (*Lolium arundinacea*) often suffer from fescue toxicosis due to ergot alkaloids produced by a symbiotic, endophytic fungus (*Epichloë coenophiala)*. These ergot alkaloids have a chemical structure similar to neurotransmitters (serotonin, dopamine, and adrenaline) and thereby mimic their physiological functions including vasoconstriction (Conolan and Taylor, 1986) and decreased circulating prolactin concentrations (Browning et al., 1997). Symptoms of fescue toxicosis in ruminants include elevated respiration rate and body temperature (Spiers et al., 2005a), decreased feed intake and average daily gain (Stuedemann and Hoveland, 1988), rough hair coat, excessive salivation, loss of circulation in extremities (Gunter et al., 2009a) and necrotic fat deposits in mesentery of abdominal cavity (Schmidt et al., 1982; Paterson et al., 1995). Fescue toxicosis related problems are exacerbated by heat stress because of impaired ability to dissipate heat due to vasoconstriction resulting in hyperthermia and reduced growth rate (Spiers et al., 2005a).

Management and nutritional approaches have been taken to reduce the toxic effects of ergot alkaloids. Some mitigation strategies, including dilution of alkaloid contaminated feed with endophyte-free forage (Roberts and Andrae, 2004) and replacement of ergot alkaloid-producing strains with a non-toxigenic novel endophyte (Bouton et al., 2002) have shown beneficial effects. Provision of compounds that produce vasorelaxation including isoflavones and estradiol have been shown to alleviate toxic symptoms (Aiken et al., 2016). Researchers have also suggested the application of yeast cell wall-based toxin adsorbents to minimize the negative effects of endophyte toxins on animal performance (Dawson et al., 2001; Wielogórska et al., 2016).

Supplementation of adsorbents in the diet has been shown to reduce the bioavailability of toxin in the GIT (Akay et al., 2003). The mean fecal total ergovaline (ergovaline and ergovalinine) concentrations of steers fed endophyte-infected tall fescue seed was significantly increased in the presence of adsorbent (yeast cell wall preparation) compared to controls, indicating a decrease in absorption of ergovaline and it's isomer from the GIT (Ely et al., 2004). Studies have also reported improved animal performance when cattle grazing endophyte

positive tall fescue were supplemented with a modified glucomannan from yeast (Ely et al., 2004; Gunter et al., 2009)

The *in vitro* evaluation of clay and yeast cell wall-based adsorbents exhibited dose-dependent interactions with ETA (Evans and Dawson, 2000). However, their adsorption specificities were lower in the presence of interfering compounds. Experiments conducted in complex media (rumen fluid and MacDougall's buffer) have shown decreased maximum adsorption capacity and affinity of adsorbents to ergotamine (Evans and Dawson, 2000). Decreased adsorption properties in the presence of interfering or competing compounds in complex media occurs when the adsorbents are nonselective in binding due to nonspecific interactions (Whitlow, 2006). Therefore, adsorbents with high specific adsorption towards target compounds are desired.

In recent years, molecular imprinting technology has gained importance for synthesis of polymers with specific adsorption through selective molecular recognition. Molecular imprinting is a technique for synthesizing macromolecular polymers with multi-functional receptor groups which aid in specific interaction with a targeted molecule (Karsten and Klaus, 2000; Sellergren and Andersson, 2000; Lian et al., 2015). These molecularly imprinted polymers (MIP) are synthesized using functional monomers, crosslinkers, initiator and porogen in the presence of an adsorbate of interest, called a template. The template interacts with functional monomers during pre-polymerization, with addition of crosslinkers to form polymers of large molecular weight (Sellergren, 2001; Cormack and Elorza, 2004). These polymers are then washed to remove the template, thereby creating open binding sites with functional groups arranged in geometries capable of binding the template and similar molecules with varying affinities (Umpleby et al., 2004). Because of specific molecular recognition properties, MIPs have been applied in various fields, especially as a sorbent material for sample clean-up in analytical chemistry (Xu et al., 2011) and as sensors in the biomedical field (Malitesta et al., 2012). Polymers made of methacrylic acid (MAA) and acrylamide have been evaluated for controlled release of phytic acid (Cirillo et al., 2010a) and sustained release of sulfasalazine (Puoci et al., 2008b) in gastrointestinal simulating fluids. In general, there is potential for application of imprinted polymers as adsorbents for toxins in the GIT because of their superior binding properties.

To date, there are no studies related to imprinted polymers as an animal feed supplement for the purpose of binding ergot toxins. As an initial step for animal nutrition experiments, custom synthesized polymers need to be evaluated *in vitro* for their adsorption properties towards targeted template and related compounds. Our research aimed at synthesizing MAA-based imprinted polymers and characterizing their morphological and adsorption properties towards template with an ultimate goal of using imprinted polymers as a specific sorbent material for ergot alkaloids.

4.2. Materials and methods

4.2.1. Reagents and chemicals

Methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA, ≥97%), 1,1,3,3-tetramethylguanidine (TMG, >97%), 2-hydroxyethyl methacrylate (HEMA, >98%), ethanol, acetone (HPLC grade) and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Fair Lawn, NJ). For purification and removing inhibitors, EGDMA, HEMA and MAA were distilled under reduced pressure and stored at 4 °C prior to use. Methanol (HPLC-grade), acetonitrile (optima grade), acetic acid and formic acid (reagent grade) were purchased from Fisher Scientific (Fair Lawn, NJ). De-ionized water (~18 MΩ) used for the analysis and preparation of solutions was obtained using a water purification system (Millipore Corporation, Bedford, MA).

2,2-Azobisisobutyronitrile (AIBN, ≥98%), ergotamine tartrate sodium salt (ETA), 2-bromo-α ergocryptine methanesulfonate salts (BC), methylergonovine maleate (ME) and lysergol (LY) were purchased in purified crystalline form (≥ 97%) from Sigma-Aldrich (St. Louis, MO). An individual standard stock solution of ergot toxins (1g·L^{-1}) was prepared in methanol and stored at -20 °C in dark until use in preparation of working solutions.

MacDougall's buffer solution containing micro- and macro-minerals along with reducing solutions were prepared as described previously (Goering and Van Soest, 1970). All chemicals (> 97% purity) required for buffer preparations including trypticase-A, sodium sulfide nano-hydrate, cysteine HCl, resazurin, ammonium bicarbonate, sodium bicarbonate, calcium chloride dihydrate, manganese chloride tetrahydrate, cobalt(II) chloride hexahydrate, ferric chloride hexahydrate, sodium phosphate dibasic, potassium dihydrogen phosphate, magnesium sulfate monohydrate, sodium hydroxide and sodium sulfide nanohydrate were purchased from Sigma-Aldrich (St. Louis, MO).

4.2.2.Polymer synthesis

The bulk polymerization with self-assembly technique was used in the synthesis of polymers. During synthesis of molecularly imprinted polymer (MIP), non-molecularly imprinted polymer (NIP) without template was synthesized using the same protocol. The ingredients used in the polymer synthesis and their chemical structure are shown in figure 4-1. Ergotamine tartrate was used as the molecular template, MAA and HEMA as functional monomers, EGDMA as the crosslinker, AIBN as free radical initiator and toluene as the porogen. A free base form of ETA was used as a template by treating with 0.6 g of TMG during pre-polymerization.

Ergotamine tartrate (0.005 mol), MAA (0.08 mol) and HEMA (0.04 mol) were dissolved in 10 mL toluene in a 250 mL round bottom flask and purged with high-purity dry nitrogen for 30 min at 22 °C. Following the incubation period, EGDMA (0.4 mol), AIBN (0.004 mol) and toluene (65 mL) were added in sequence. Polymerization was initiated by placing the reaction mixture in an oil bath set at 65 °C and propagated for 5 h with stirring. Polymerization was terminated by reducing the temperature to 22 °C. The mixture was then filtered and the pellet was subjected to template removal procedure.

Template removal was carried out by sequential washings of the pellet with 100 mL of three different solvents (methanol (5x), acetonitrile (5x) and DI water (1x)) with vigorous shaking (15 min) and sonication (15 min) followed by centrifugation (4000 g, 30 min) and filtration. The filtrates were analyzed for ETA concentration and total recovery of the template was calculated. Washed polymer was freeze-dried under vacuum (<100 mT, – 46 °C, 48 h) and oven dried (120 °C, 1 h) before the net weight was determined. Dried polymer was then ground to fine powder using mortar and pestle, and sieved using standard metal sieves (VWR- USA Standard Testing Sieves) to obtain a < 250 μm particle fraction.

4.2.3.Morphological characterization of polymer

4.2.3.1. Light scattering diffraction

Morphological characteristics of polymers were assessed by light scattering diffraction (DLS) using UV laser light source operated at a wavelength of 375 nm with continuous wide-angle dynamic light scattering setup (SALD- 7101, Nanoparticle size analyzer, Shimadzu Scientific instruments, Columbia, MD). Samples were suspended in DI water (0.1 $g \cdot L^{-1}$) and sonicated (1

min) before measurements were taken. To reduce aggregation, samples were prepared by suspending polymer in 5% aqueous methanol. The instrument was set to measure between 0.5 nm and 300 µm at 21 ± 0.1 °C and all measurements were made in triplicate, with each replicate measured 15 times.

4.2.3.2. Scanning electron Microscopy

Morphological characteristics were investigated by scanning electron microscopy (SEM: Hitachi S-4300, Tokyo, Japan). The sample was mounted on a pin mount (aluminum) and coated with gold particles using a sputter deposition system (38 A°/min) for 3 min under vacuum and at 3 kV at a working distance of 9.8 mm.

4.2.3.3. Surface area and porosity

Structural properties of polymers were characterized using nitrogen adsorption/desorption isotherms at -196.15 °C according to the Brunauer-Emmett-Teller (BET) procedure (Brunauer et al., 1938). Polymers were evaluated for pore size distribution, pore volume and specific surface area. A 15.0 mg sample was heat treated (100 °C) for 3 hours under inert gas flow to remove atmospheric contaminants. The sample was then cooled, degassed under vacuum (1 x 10^{-5} Torr) and exposed to increasing nitrogen gas pressures. Nitrogen gas adsorption/desorption was measured under cryogenic conditions using an Autosorb-1C (Quantachrome Instruments, FL, USA) gas sorption analyzer. The BET surface areas were calculated from the adsorption isotherms in the relative pressure range from 0.06 to 0.2 psi. Pore size distribution and total pore volume were determined using the Barrett-Joyner Halenda (BJH) method (Barrett et al., 1951).

4.2.4.Evaluation of polymers

4.2.4.1. Isotherms adsorption studies

Equilibrium isothermal adsorption studies were conducted to determine the adsorption properties of polymers to ergotamine tartrate. All experiments were conducted in silanized 250 mL amber colored screw cap bottles in triplicate. Polymers (0.1 mg·mL^{-1}) in 100 mL MacDougall's buffer (pH 6.8) were exposed to increasing concentrations of ergotamine tartrate (0.15, 0.76, 1.52, 7.61, 15.23, 22.84, and 30.45 µmol·L^{-1}) and incubated on a horizontal shaker (150 rpm, 39 °C, 1.5 h). A 1.5 mL aliquot sample was centrifuged and the supernatant (1 mL) was transferred

to silanized amber color HPLC vials (Waters Corp., Milford, MA) for high performance liquid chromatography-fluorescence detection (HPLC-FLD) analysis of ETA at equilibrium. Amount adsorbed ($\mu mol \cdot g^{-1}$) was calculated from the difference in concentration between the samples and their corresponding controls (without polymer) at each concentration.

Isothermal adsorption properties of the polymer towards ETA was analyzed by fitting the data to non-linear Langmuir (Langmuir, 1918) and Freundlich (Freundlich and Hatfield, 1926) models (Table 4-1) using GraphPad Prism (version 5; GraphPad Software Inc., La Jolla, CA). The fits were ascertained using correlation coefficients and lowest residual variance. Model selection was based on absolute sums of squares of residuals and the probability of selecting one model over other, determined using bias-corrected Akaike's Information Criterion (AICc) for the two models (GraphPad Prism Software, version 5.0, USA). The AICc is a measure for determining the validity of model selection within a cohort of nonlinear models (Burnham and Anderson, 2003; Spiess and Neumeyer, 2010).

4.2.4.2. Selectivity experiment

Batch isothermal adsorption was conducted in MacDougall's buffer, pH 6.8, containing four ergot alkaloids (ETA, BC, ME, and LY) in the same solution. A mixture of ergot alkaloids (initial concentration of each alkaloid: 12.18 $\mu mol \cdot L^{-1}$) was prepared in the buffer and serially diluted (5x) by a factor of two to obtain five levels of the alkaloids. MIP and NIP (0.1 $g \cdot L^{-1}$) were exposed to increasing concentrations of alkaloid mixture (0.76, 1.52, 3.04, 6.09 and 12.18 $\mu mol \cdot L^{-1}$) for 90 min at 39 °C. A control was maintained without the polymer at similar alkaloid mixture concentrations. The samples were centrifuged (14,500 x *g*, 10 min) and the supernatant was analyzed for alkaloids using HPLC-FLD. Adsorption coefficients (*k*) towards each of the alkaloids in the presence of different alkaloids was determined using the following equation

$$k = \frac{[Co - Ce]}{M} \times V$$

where, *Co* and *Ce* are initial and final equilibrium concentration of ergot alkaloids ($\mu mol \cdot L^{-1}$), *V* is the volume of adsorption medium in liters, and *M* is the amount of polymer used in the medium (mg). Selectivity for the adsorption of ergotamine tartrate in the presence of interfering compounds was estimated by selectivity coefficient (*k'*) using the equation:

$$k' = \frac{k(ETA)}{k(alkaloid)}$$

where *k(ETA)* is the adsorption coefficient of ETA and *k* (alkaloids) represents the adsorption coefficient of each of the three structurally related alkaloids tested (BC, ME and LY).

The relative selectivity coefficient (*k'')* for each alkaloid, which determines the effect of imprinting on selectivity, was estimated by the ratio of *k'* values of the imprinted polymer to the non-imprinted polymer as follows:

$$k'' = \frac{k'\,imprinted}{k'non - imprinted}$$

4.2.4.3. Molecularly imprinted solid phase extraction (MISPE) study

Polymers were evaluated for their adsorption efficiency using solid phase extraction. Polymers were added as a sorbent material in solid-phase extraction (SPE) columns and were compared for their adsorption efficiency to a commercially available C_{18} SPE column. Polymers (100 mg) were packed between two quartz frits in 7 mL solid-phase extraction reservoirs (Alltech, Deerfield, TI) in triplicate. The columns were primed with 5 mL of methanol followed by 5 mL of MacDougall's buffer (pH 6.8) and air dried before loading with 5 mL of MacDougall's buffer spiked with ETA (0.1, 1 10 and 20 $mg \cdot L^{-1}$). The spiked sample was allowed to percolate through the column for 10 min and later was pushed slowly at a flow rate of 0.5 $mL \cdot min^{-1}$ and the eluent was collected. The MISPE column was then washed with 5 mL of MacDougall's buffer followed by 5 mL of extraction solvent (methanol: acetic acid, 9:1, v/v) to remove any loosely bound ETA and to extract ETA, respectively. The effluent from the columns was dried under nitrogen at 35 °C and reconstituted in acetonitrile (1 mL) for analysis of ETA by HPLC-FLD.

The utility of the MISPE column for sample clean-up and for extraction of ergot alkaloids from fescue seed was determined. Briefly, endophyte free fescue seed sample was ground and spiked with ETA (1 $\mu g \cdot g^{-1}$, dry matter basis) and shaken on an orbital shaker for 30 min. For extraction, spiked fescue seed (20 g, n=3) was suspended in 50 mL alkaline methanol (0.1M NaOH in methanol) and incubated on a horizontal shaker (250 rpm, 20 min, 22 °C). A 20 mL aliquot was dried under a nitrogen stream and reconstituted in 20 mL of MacDougall's buffer of

pH 6.8. To evaluate the extraction efficiency, 5 mL of sample was loaded to MISPE column and pushed at a flow rate of 0.5 mL·min^{-1}. The columns were then washed with 5 mL of MacDougall's buffer (pH 6.8) followed by 5 mL methanol. The effluents from the column were collected, dried under nitrogen (35 °C), reconstituted in 1 mL of acetonitrile and analyzed for ETA by HPLC-FLD.

4.2.5.Fourier Transformed Infrared (FTIR)

Polymers and polymer+ETA complex were evaluated for functional groups by Fourier transformed infrared spectrometry (100 FTIR spectrometer, Perkin-Elmer, Waltham, MA. USA) according to procedures described for assessing polymer interactions (Molinelli et al., 2005). A 10 mg sample was placed on a diamond crystal and absorbance was recorded at a frequency range (4000-600 cm^{-1}) for qualitative examination of vibrational frequencies of functional groups (C-C, C-H, C=C, O-H, N-H). For each measure, 10 scans with a resolution of 2 cm^{-1}, with automatic baseline correction were performed.

4.2.6.High performance liquid chromatography-fluorescence detection (HPLC-FLD) analysis

A series 2695 Alliance HPLC separation module (Waters, Milford, MI) equipped with a binary pump, an auto sampler, column oven and a fluorescence detector (474 scanning fluorescence detector, λ_{ex} 250nm, λ_{em} of 420nm, gain 16 and attenuation 1000) was used to measure ergot alkloids in the samples. Chromatographic data were integrated using Waters Empower 3 software 7.00.00.99 (Waters, Milford, MI). HPLC separations were performed with a 100 x 4.6 mm i.d., 2.6 µm particle size, Kinetex C_{18} column (Phenomenex, Torrance, CA) with a gradient elution consisting of two mobile phases, A) water and B) acetonitrile, both spiked with ammonium hydroxide (0.04%). Initial gradient conditions were 100% A held for 1 min, increasing linearly to 100% B over 12 min and held for 3 min. The processed terminated with a linear return to initial conditions over 3 min, which was then held for 2 min for a total run time of 21 min. The sample injection volume was 50µL. Samples were evaporated to near dryness and the residue dissolved in methanol/water (50/50) and analyzed (Rottinghaus et al., 1991).

4.2.7.Statistical analysis

Results of *in vitro* adsorption studies were analyzed as completely randomized design with the PROC GLM procedure (SAS Inst. Inc., Cary, NC). The model included concentration of

analyte, type of analyte, type of polymer, and the interaction of analyte concentration × type of analyte. Least squares means were calculated for analyte concentration, type of analyte, and the analyte concentration × type of analyte interaction. The adsorption models were compared using AICc for the best fit model (Spiess and Neumeyer, 2010) using the option in GraphPad (GraphPad Prism Software, version 5.0, USA). The experimental unit was an individual vessel. Within each analysis, means were separated using protected Fisher's least significant differences and differences were considered significant at $P < 0.05$.

4.3. Results and discussion

4.3.1.Polymer synthesis

MAA and HEMA based copolymers were synthesized by bulk polymerization with ETA as a template. Functional monomers like methacrylic acid are commonly selected for polymer synthesis to mimic biological interactions because of their anionic acid functional groups which allow non-covalent interactions. Additionally, with a non-covalent imprinting approach, it is easy to fabricate the polymer, which helps in template removal (washing) and rapid template rebinding (Mosbach, 2006a).

The concentration of initiator was 0.03 mol per mole of monomers, which led to a high conversion rate of monomers to polymer (95 % conversion within 4 h). Reaction time was shorter than reported for similar studies using MAA monomers and free radicle initiation using AIBN, with different templates where reactions were run overnight or for 24 h (Baggiani et al., 1999; Nantasenamat et al., 2006; Alsudir and Lai, 2012). Conversion rates between 83 and 93% with MMA monomers and AIBN when using a ratio of 1:0.003 moles was observed previously (Lyoo et al., 2004). EGDMA was chosen as the crosslinker because of the four polymerizable functional groups it can present for interaction. Crosslinkers provide structural rigidity to polymers and play an important role in maximizing the quality of imprinting (Strikovsky et al., 2000). The ratio of template to monomer to crosslinker plays an important role in the selectivity of adsorption of the polymer towards the template. For an MAA-based polymer that was imprinted with an aromatic template, better selectivity was observed when the ratio of template: monomer: crosslinkers was 0.4:1.4:5.6 (Nantasenamat et al., 2006). In another study, metergoline templated, MAA and EGDMA-based MIP in the ratio of 1:4 showed better binding properties and selectivity towards ergot alkaloids (Lenain et al., 2012a). Therefore, in our study,

the ratio of 0.06: 1.5: 5 (template: monomer: crosslinker) was chosen to in order to obtain an imprinted polymer with good specificity.

Ergotamine tartrate, an ergopeptide alkaloid, was chosen as a template to represent ergot alkaloids. The tetracyclic ring is a common structural component of all ergot alkaloids, however, those containing peptide side chains (ergopeptides) are considered more toxic than ergolines (Egert, 2014; Pesqueira et al., 2014) since they induced stronger contractile responses in myographic studies. Studies have shown that MIP can selectively interact with compounds that are structurally similar to the template and therefore can be used to adsorb compounds that shares the structural properties (Weiss et al., 2003; Krska et al., 2005; Baggiani et al., 2007). In our study, MIP were synthesized using commercially available ETA as template, with the intent that they should selectively interact with compounds having a tetracyclic ring (ergolines) and tripeptide side chains (ergopeptides).

Nearly 31.8 grams of polymer was synthesized with a polymerization conversion percentage of 95% of all chemicals used. Quantitatively, 1.38 g (38%) of the template was recovered in porogen (toluene) filtrate indicating that the remaining 2.04 grams was utilized in imprinting of polymer. MIP washings with methanol (five washes) recovered 99% of the template that interacted with polymer which could be attributed to high solubility ($2 mg \cdot mL^{-1}$) of ETA in methanol (Remington et al., 2006).

4.3.2.Polymer morphology

4.3.2.1. Particle size distribution: Light scattering diffraction

Figure 4-2 shows the hydrodynamic diameters of MIP and NIP used in our study determined by DLS. Even though the range of particle size distribution is a function of polymerization steps (initiation, propagation and termination) described by the power law (Matyjaszewski and Davis, 2002), mechanical grinding and swelling properties of the polymer in different solvents contributed to a wide range of particle size distribution. The hydrodynamic diameters for both the polymers ranged between 5 and 100 µm irrespective of solvent except for NIP in water where the particle size ranged between 50 and 250 µm. The maximum particle size was not more than 250 µm since the polymer was screened through < 250- µm sieve. The final particle size distribution reflected the effect of grinding and the inherent swelling

properties of the polymers along with aggregation due to hydrophobic interactions in aqueous media.

4.3.2.2. Scanning electron Microscopy

The SEM micrographs of MIP and NIP in their dry form revealed non-uniform dispersion of particles with irregular morphologies (Figure 4-3). NIP had a more regular structure than MIP. The surface of MIP exhibited more cavities than NIP, probably caused by the molecular structure of ETA, suggesting changes in structural properties and network organization of MIP due to the presence of the template (Kudupoje, 2014).

4.3.2.3. Surface area and porosity

Nitrogen absorption increased with increase in relative pressure due to capillary condensation /evaporation of nitrogen into pores of the polymer. Both polymers exhibited responses typical of microporous particles (Figure 4-4). The BET surface area for MIP (254 $m^2 \cdot g^{-1}$) was three times greater than NIP (82 $m^2 \cdot g^{-1}$). Additionally, a similar trend in micropore volume was observed, which was evident by BJH adsorption values showing cumulative pore volume of 0.022 and 0.006 $cm^3 \cdot g^{-1}$ (table 4-2) for MIP and NIP, respectively. However, the pore diameter was greater for NIP than MIP by a factor of two. The comparatively large BET surface area and pore volume of MIP were presumably created when the imprinting template molecule was removed from the polymer.

4.3.3.Evaluation of polymers

4.3.3.1. Isotherm adsorption studies

Adsorption studies were conducted in MacDougall's buffer that represented the mineral composition of ruminant saliva. A negative control without ETA was maintained to account for template bleeding to prevent potential error in adsorption data. Both polymers exhibited increasing dose-dependent adsorption to increasing concentration of ETA in solution (Figure 4-5). Langmuir and Freundlich isotherms were used to quantitatively describe adsorption properties of polymers to ETA (Table 4-3).

Langmuir and Freundlich are two parametric models that evaluate monolayer homogeneous adsorption of identical adsorption sites with similar energies and multiple layer heterogeneous surface adsorption on the adsorbent, respectively (Langmuir, 1918; Vermeulan

et al., 1966; Hutson and Yang, 1997). Both models had correlation coefficients of >0.92 for both polymers, which reveal the applicability of these isotherms. The validity of the nonlinear regression models indicated by the AICc showed a better fit for both the polymer with Langmuir (> 99.5% probability of being correct) compared to Freundlich model (0.39 – 0.47% probability of being correct). Additionally, for both polymers, the absolute sum of squares was lower with Langmuir (33.6 and 14.43 for MIP and NIP, respectively) compared with Freundlich model (56.08 and 26.71 for MIP and NIP, respectively). The residuals were randomly scattered around zero indicating that the model reasonably describe the data and normal probability plots suggested that the random errors affecting the adsorption process were normally distributed.

A better fit with Langmuir models suggested a monolayer adsorption on the surface of both the polymer. The Langmuir constant (R_L) refers to favorability (Nwabanne and Igbokwe, 2008) and energy of adsorption (Weber and Chakravorti, 1974) which depends on the molar activity coefficient of occupied and unoccupied sites on the adsorbents at equilibrium (Graham, 1953). The R_L value is a dimensionless constant which predicts the affinity of the adsorbate to the adsorbent and values between 0 and 1 indicate favorable adsorption. The R_L values in the present study were between 0.21 and 0.39 for both polymers, indicating adsorption between ETA and polymer was favorable. The maximum adsorption capacity (Qo) occupying a monolayer surface of the polymer was higher for MIP (15.83 ± 1.24 $\mu mol \cdot g^{-1}$) compared to NIP (12.37 ± 0.75 $\mu mol \cdot g^{-1}$). Compared to our previous studies, the capacities of MAA based polymers were higher than styrene based polymers (8.68 $\pm$ 0.80 and 7.55 $\pm$ 0.61 $\mu mol \cdot g^{-1}$ for MIP and NIP respectively), but there were no difference in affinities.

The interaction between the template and polymer is a complex process that is governed by several factors including ratios of reactive functional groups, imprinting effect that created structural complementary voids and solvent effects (Svenson et al., 2004). A larger monomer: template ratio has been shown to increase the number of selective sites (Yilmaz et al., 1999) but compromised its specific adsorption properties (Sellergren, 1997). In our study, using a copolymerization of MAA with HEMA, there are possibilities that excess of interaction sites was created irrespective of the imprinting effect. In the case of ETA as template, the favorable interaction with the active functional monomer could be mainly due to the presence of weak hydrogen-bonding, although other types of molecular interactions could influence the adsorption process.

4.3.3.2. Selectivity experiment

The selectivity of MIP was evaluated by adsorption studies with structurally related compounds and by comparing MIP to NIP. The binding capabilities of each template analog towards polymer were characterized using adsorption coefficients which account for adsorption selectivity. For each of the 4 alkaloids evaluated, adsorption differences between MIP and NIP depended on both the alkaloid type and the initial concentration ($P < 0.01$), however, the product differences were generally consistent across concentrations (Figure 4-6). With BC and ME, average adsorption (of five concentrations tested) were significantly greater for MIP than NIP (62.9 vs 37.1 and 35.8 vs 24.7%, respectively, $P < 0.01$) and no difference ($P > 0.05$) existed between polymers for ETA and LY adsorption.

Table 4-4 summarizes selectivity parameters (*k*, *k'* and *k''* values) for BC, ME, and LY adsorption compared to ETA for MIP and NIP. Both polymers had similar *k* values for binding of ETA, indicating that the polymers did not differ in selectivity for ETA. Both polymers had low k values to BC and LY, however, MIP showed higher adsorption to ME compared to NIP. In reference to selectivity coefficient, (*k'),* higher *k'* value indicates better selectivity of polymer towards rebinding of template (ETA). Comparing *k'* values between polymers, it was evident that MIP selectively bound ETA compared to BC or LY. Even though similar trend existed with NIP in selectivity (*k'*) towards BC and LY, *k'* values of NIP were generally lower compared to MIP indicating relatively lower selectivity in adsorption, except for ME. The imprinting effect (*k'')*, is intended to evaluate the imprinting effect, where higher coefficients indicate greater selectivity towards the template. Although k'' values were slightly above 1 for BC and LY, these ratios were the consequence of small differences between MIP and NIP at comparatively low levels of alkaloid binding. The most salient difference between polymers was the greater binding affinity by MIP for ME, suggesting non-selective binding by MIP, which could mainly be due to the higher surface area of that polymer. As a large ergopeptide molecule (15.71 A^{o}; www.chemspider.com/Chemical-Structure), ETA may form larger binding voids during the imprinting process thereby creating a favorable access for smaller ergoline molecules like ME (maximum molecular size was < 8.82A^{o}; www.chemspider.com/Chemical-Structure), which can possibly interact with the active functional component of the polymer. However, this geometrically defined adsorption site created through the interaction of template with the functional monomers was not favorable to BC and LY, which could be due to hindrance in access

to the adsorption site or a mismatch in alignment of functional groups and the structural complement. This study indicated that both polymers could bind ergoline and ergopeptide alkaloids. However, the differences in the adsorption properties could be attributed to the surface area differences along with some changes in structural properties between the MIP and NIP created during the synthesis of MIP, which could provide additional functional groups for interactions with the molecules.

4.3.3.3. Molecularly imprinted solid phase extraction (MISPE) study

Polymers were evaluated as sorbents in solid-phase extraction (SPE) columns to determine their application as a specific adsorbent material for ETA. Commercially available C_{18} SPE cartridges are polymerically bonded octadecyl compounds commonly used for pre-concentration or extraction of organic molecules. An MIP-made SPE cartridge of 100mg capacity was evaluated and compared to commercially available SPE filtering. While preparing MISPE cartridges, quartz frits were used to hold the polymer in the column to reduce non-specific adsorption of ETA to PTFE frits as previously characterized (data not shown). Preliminary studies showed that PTFE frits significantly bound ETA molecules leading to biased data. On the other hand, the large number of functional groups provided by different monomers (MAA, HEMA and EGDMA) in co-polymerized products also contribute to weak non-specific binding (Qi et al., 2012). To account for nonspecific interaction, the columns were washed with the loading solvent. The washing fractions were collected and analyzed for ETA concentration. A second wash with methanol: acetic acid (9:1, v/v) was performed to extract ETA from the columns to give insight on the strength of interactions between the polymer and its applicability to ETA sorption. The amounts of initial adsorption and the amount retained in the sorbent materials following a buffer and a solvent wash are shown in figure 4-7. All the adsorbents retained more than 99% of the initial amount of ETA loaded, except at the lowest concentration (0.1 $\mu g \cdot mL^{-1}$), where 94 to 95% of the ETA was retained, irrespective of polymer type. No difference was observed between polymer type ($P = 0.23$). The percent retention after buffer wash was significantly lower ($P < 0.01$) for NIP compared to MIP and C_{18}, however, there was no difference ($P = 0.32$) between MIP and C_{18}. After the solvent wash, the percent ETA that remained on the NIP was significantly lower ($P < 0.03$) than the two other polymer types, accounting for larger non-specific interactions.

In a second MISPE experiment, polymers were evaluated for their application as sorbent materials in SPE column, either for sample clean-up or pre-concentration of ETA from a complex biological matrix. Ground fescue seed (alkaloid free) sample spiked with ergot alkaloids ($1\mu g \cdot g^{-1}$, ETA, LY, ME) was used. Results from this study showed similar retention properties to evaluation carried out in buffer media (Figure 4-8). Comparing different alkaloids, there was no difference ($p=0.40$) between products in their initial retention. After the first wash with buffer, MIP and C_{18} had significantly higher ($P < 0.01$) adsorption to ETA and LY compared to NIP. In addition, MIP had significantly higher retention properties ($P < 0.01$) towards all three alkaloids after the third wash with extraction solvent compared to NIP and C_{18}, indicative of a stronger interaction with alkaloids in the presence of the matrix from fescue seed.

In both studies using MISPE, there was no difference between the MIP and NIP in the initial retention to the polymers. Although more attention has been focused on noncovalent imprinting (Cormack and Mosbach, 1999), especially for aromatic templates and MAA-based polymers (Sikiti et al., 2014; Zarejousheghani et al., 2014), because of binding properties (Ramström et al., 1996; Mosbach, 2006), on a per bond basis, non-covalent bonds are 1 to 3 order of magnitude weaker than covalent bonds leading to a large number of heterogeneous and different-affinity adsorption sites (Elemans et al., 2003). The recognition of phenol via hydrogen bonding is suppressed in water, while hydrophobic interactions, though promoted, are not specific enough for highly-selective phenol recognition. Such contradictory effects have been observed previously in imprinted polymers targeting penicillin antibiotics (Cederfur et al., 2003). Acrylate-based imprinted polymers are generally synthesized using the non-covalent polymerization approach and are known to interact via H-bonding, van der Waals, electrostatic, and hydrophobic interaction (Andersson et al., 1984; Vlatakis et al., 1993; Venkatesh et al., 2007). The presence of several adsorption mechanisms may have led to a high degree of non-specific interactions, irrespective of polymer, which could have led to the absence of difference between the MIP and NIP in initial adsorption. Eliminating non-specific adsorption through buffer washing indicated better adsorption to MIP.

4.3.4.Fourier Transformed Infrared (FT-IR)

The FTIR spectra of the MIP and [MIP + ETA] complexes are presented in Figure 4-9. The spectra reflect the complex nature of the polymer and show significant band shifting and

intensity changes due to interaction with ETA. The major absorption bands observed were at 3383 cm^{-1} (–OH and –NH groups), 2931 cm^{-1} (asymmetric stretches of –CH group), 1704/ 1461 cm^{-1} (C=C and C=O bonds) and 1118/ 1086 cm^{-1} (C–O stretching) providing potential interaction sites on the polymer.

Absorption above 1400 cm^{-1} was shifted or diminished which indicates the involvement of functional groups in the adsorption process. The spectrum attributed to –OH and –NH groups (3200-3600 cm^{-1}) were decreased in intensity in [MIP+ETA] complex indicating the involvement of hydroxyl group and an amine group in the adsorption process via hydrogen bonding. New peaks were also detected in the complex [MIP + ETA] due to the presence of ETA. The change in intensity of spectrum between 3000cm^{-1} and 2931 cm^{-1} accounted for a change in vibration of Csp2-H and Csp3-H, which could be indicative of an interaction between the ETA and PMAA via hydrogen bonding. The appearance of absorption at 1700 cm^{-1} in PMAA+ETA complex sample could be attributed to the C=O contributed by ETA in the complex. The intensity of C-O stretch (1118 cm^{-1}) and C-H bend of CH2 (1461 cm^{-1}) diminished in MIP+ETA sample which indicates a change in vibrational energies after the formation of the complex.

4.4. Conclusion

A molecularly imprinted polymer (MIP) with ETA as templates for the selective recognition of ergot alkaloids was synthesized using MAA and HEMA as functional monomers. It appears that the amine, hydroxyl and carbonyl moiety of ETA has the potential to interact with hydroxyl group of HEMA or the carbonyl functional groups of the MAA monomers. Moreover, the presence of the ester carbonyl groups in the EGDMA crosslinker may lead to the formation of additional hydrogen bonds with the hydroxyl groups of the ETA. Our study demonstrated that the use of the non-covalently imprinting polymer is a valuable approach in the imprinting of analytes that are not soluble in polar solvents, as the hydrogen bonding interactions are favored in this aprotic porogen. The resulting hydroxyl-functional P(MAA-co-HEMA) copolymers offer a variety of new possibilities for the preparation of a variety of novel PMAA-based macromolecular architectures.

Compared to experiment 1, the presence of hydroxyl groups from P(MAA-co-HEMA) co-polymer favors interaction in aqueous media better than the styrene based polymers, which is indicated by higher adsorption capacities values. The adsorption coefficient of the MIP and NIP

for their template did not differ, which contradict the high selectivity described for the imprinted polymers. It should be noted that unlike other research, most of our isothermal adsorption studies were conducted in buffered aqueous media, where hydrogen bonding is suppressed and interactions like van der Waals, electrostatic, and hydrophobic are favored. Those favored interactions are generally common between MIP and NIP leading to little difference in adsorption between polymers (Gryshchenko and Bottaro, 2014).

The presence of several functional groups in the template that has the potential for complex interactions may complicate generalizing the adsorption results to either polymer (NIP or MIP). It was evident that MIP had better strength in adsorption towards ergot alkaloids compared to NIP. Additionally, the imprinted polymer was capable of adsorbing ergot alkaloids with greater specificity in the presence of complex matrix like fescue seed samples. If the application of such polymeric material can be foreseen for the specific extraction or filtration from fescue seed, to further isolate ergot alkaloids and to enhance, for example, recoveries as an application in analytical chemistry, this polymeric material could also be investigated in the context of mitigating the impact of ergot alkaloids in production animals that are often exposed to them. As such, this polymeric materials or more organic alternatives, could also be investigated, depending on their composition, as feed additives provided that they fit with compositions defined as safe to be fed to animals.

The found better adsorptive properties in aqueous media of the imprinted polymers would be as such more compatible for their application as feed additives that potentially could reduce the bioavailability of ergot alkaloids in the GIT environment. Application however of styrene based polymers as feed additive may require additional studies to determine their compatibility with biological systems. On the other hand, MAA based polymers are more widely researched in biomedical field as drug delivery systems and may better feed additives candidates compared to styrene based polymers. In the event of application of MAA-based polymers as feed additives to mitigate the toxic effects of ergot alkaloids, there are few important concerns that needs to be addressed, especially for ruminants. From a nutritional stand point, polymers (as additive) should not interfere with ruminal fermentation and should not have high nonspecific cross-interaction with minerals, vitamins or amino acids that could lead to performance decrease or health issues. They should bind ergot alkaloids in rumen and preserve this interaction along the downstream gut conditions. Polymers should be stable

enough to survive digestion, and not degrade into toxic residues. Therefore, determining the effect of polymers on ruminal fermentation was deemed necessary to ascertain that polymers are inert enough to potentially be used as feed additives.

Table 4-1. Two parameter Isothermal adsorption models.

Model	Equation	Parameters
Langmuir	$q_e = \frac{Q_o K_L C_e}{1 + K_L C_e}$	q_e: amount adsorbed, µmol·g^{-1} K_L: Langmuir adsorption constant Q_o: maximum amount adsorbed (µmol •g^{-1}) C_e: is equilibrium concentration of adsorbate (µmol.L^{-1})
	$R_L = \frac{1}{1 + K_L C_e}$	R_L is Langmuir isotherm constant, K_L: Langmuir constant
Freundlich	$\mathrm{qe} = \mathrm{K_f C_e}^{\left(\frac{1}{\mathrm{n}}\right)}$	K_f: adsorption capacity factor 1/n indicates adsorption intensity or surface heterogeneity index

Table 4-2. Physical properties of NIP and MIP from BET and BJH analysis.

	MIP	NIP
BET surface area, m^2/g	253.966	81.79
Volume : Micropores, cm^3/g	0.022	0.006
Average pore diameter, (4V/A), nm	3.529	6.208

Table 4-3. Isothermal adsorption constants of two isotherms models for ergotamine tartrate adsorption onto MIP and NIP.

	MIP	NIP
Langmuir Isotherm Constants		
Q_o (µmol·g^{-1})	15.83 ± 1.24[a]	12.37 ± 0.75[a]
K_L (L·g^{-1})	0.362 ± 0.09[a]	0.819 ± 0.21[a]
R_L	0.39[a]	0.216[a]
R^2	0.945	0.961
Freundlich Isotherm Constants		
K_f (µmol·g^{-1})	4.863 ± 0.45[a]	5.366 ± 0.44[a]
1/n	0.423[a]	0.322[a]
n	2.359 ± 0.05[a]	3.103 ± 0.04[a]
R^2	0.95	0.92

q_e: amount adsorbed, µmol·g^{-1}; b or K_L: Langmuir adsorption constant; Q_o: maximum amount adsorbed (µmol •g^{-1}); C_o: initial concentration of adsorbate (µmol.L^{-1}); C_e: is equilibrium concentration of adsorbate (µmol.L^{-1}); K_f: adsorption capacity factor and 1/n: adsorption intensity or surface heterogeneity index.
Data represent mean standard error of mean (n=3).
Values with different superscript within the column are significantly different (P <0.05)

Table 4-4. . Selectivity parameters of the MIP and NIP.

	MIP MAA		NIP MAA		Imprinting effect,
Alkaloids	*k* [1]	*k'*	*k*[1]	*k'*	*k''*
Ergotamine	0.990 ± 0.004[a]		0.988 ± 0.006 [a]		
Methylergonovine	0.853 ± 0.019[a]	1.162 ± 0.027	0.146 ±0.019 [b]	6.976 ± 0.812	0.172 ± 0.022
2-bromo-alpha-ergocryptine	0.146 ±0.019 [a]	6.992 ± 0.814	0.176 ± 0.014 [a]	5.677 ± 0.487	1.248 ± 0.098
Lysergol	0.283 ± 0.037 [a]	3.612 ± 0.461	0.332 ± 0.031 [a]	3.031 ± 0.286	1.212 ± 0.116

Data represent the mean affinity value ± standard error of mean (n=3)

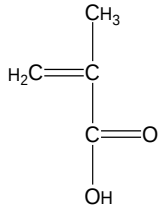
k : adsorption coefficient; k': selectivity coefficient; k'' : effect of imprinting on selectivity

[1]*k* values with different superscripts differ ($P < 0.01$) between MIP and NIP within a given alkaloid.

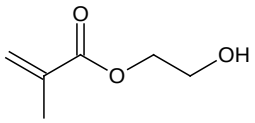
Polymer type x alkaloid type interacted ($P < 0.01$)

Monomers

Methacrylic acid

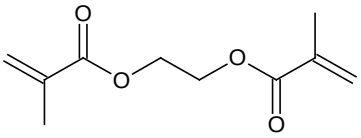


Hydroxyethyl methacrylate



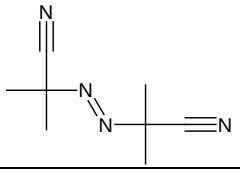
Crosslinker

Ethylene glycol dimethacrylate



Initiator

2,2'-Azobisisobutyronitrile



Template

Ergotamine tartrate

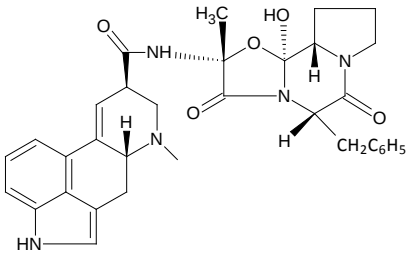


Figure 4-1. Chemical structures of the ingredients that were used in the synthesis of the polymer.

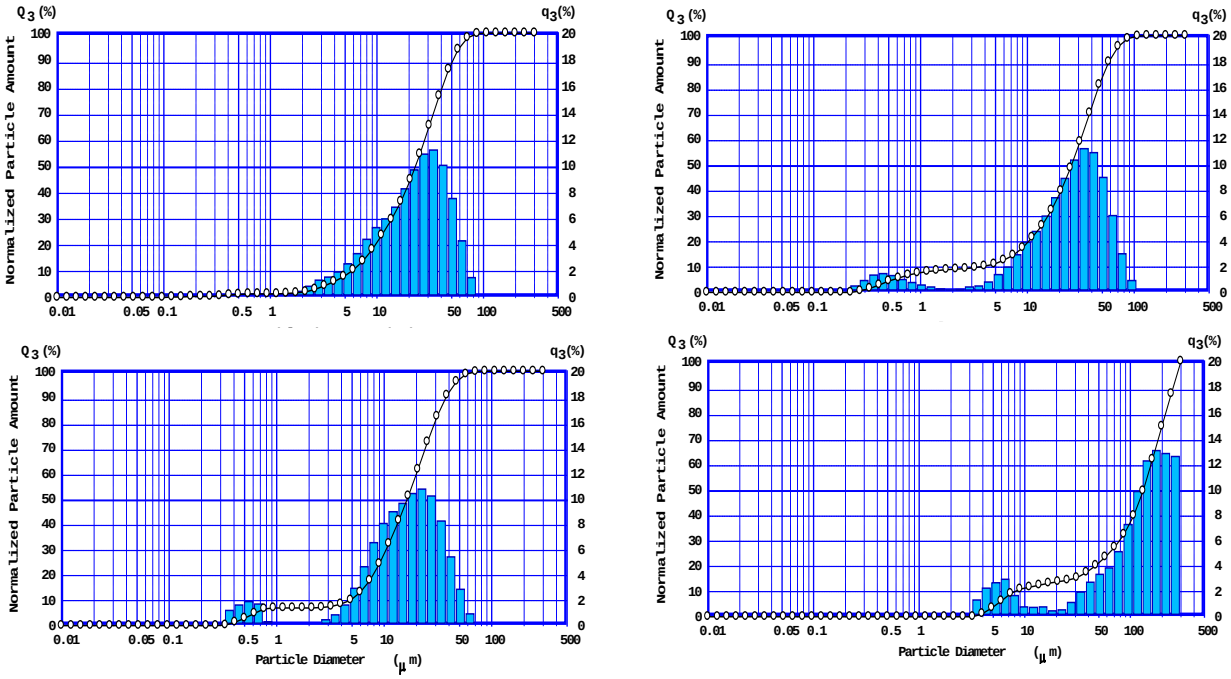
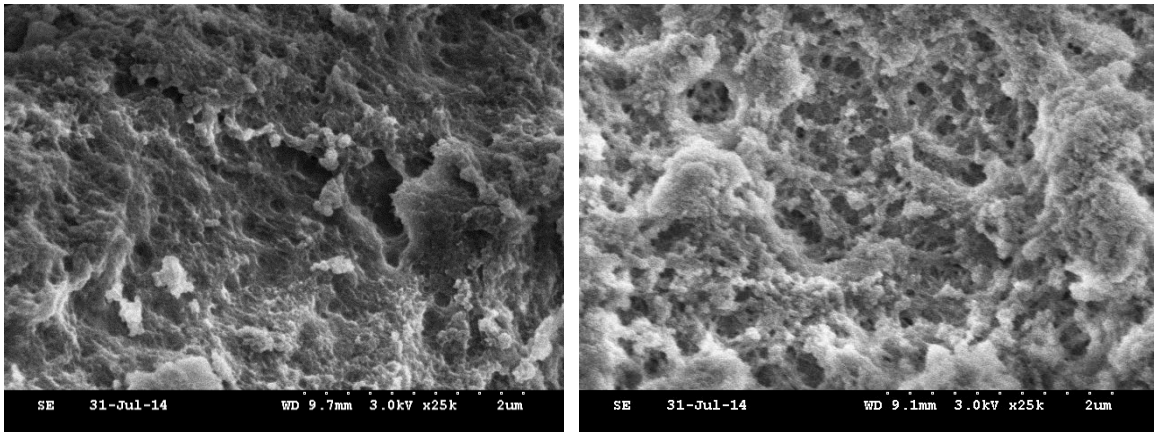


Figure 4-2. Particle size distribution of MIP and NIP in 5% methanol (1a and 1b) and water (2a and 2b).



MIP NIP

Figure 4-3. SEM of MIP and NIP (25K magnification).

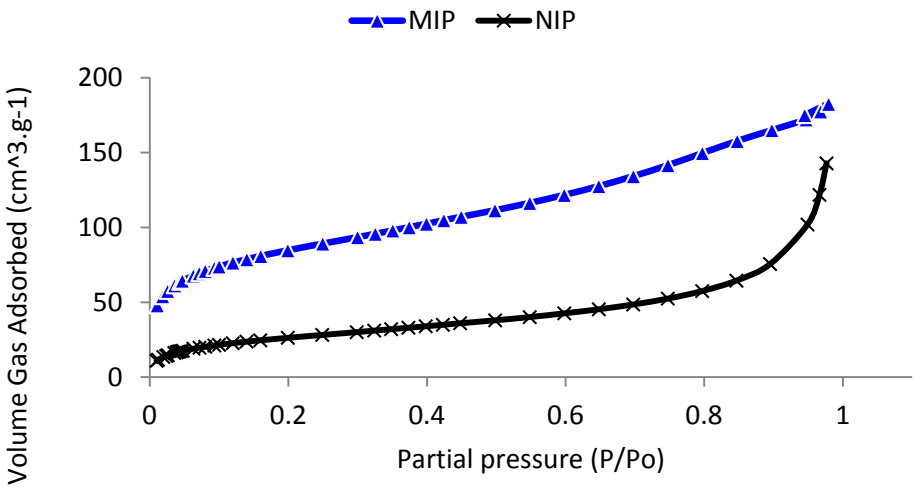


Figure 4-4. Isothermal adsorption of N2 gas onto NIP and MIP Particles to determine the surface area.

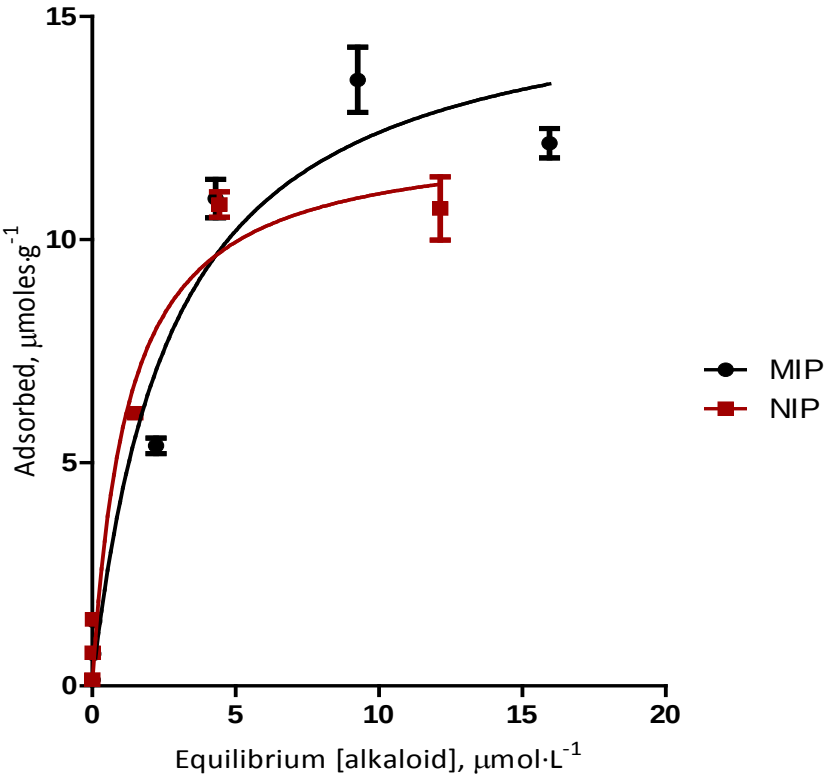


Figure 4-5. Langmuir isothermal adsorption plot of the bound concentration of ETA (µmol·g^{-1}) to MIP and NIP as a function of the concentration of free ETA at equilibrium. The data in the figure represents mean ± standard error of mean (n=3).

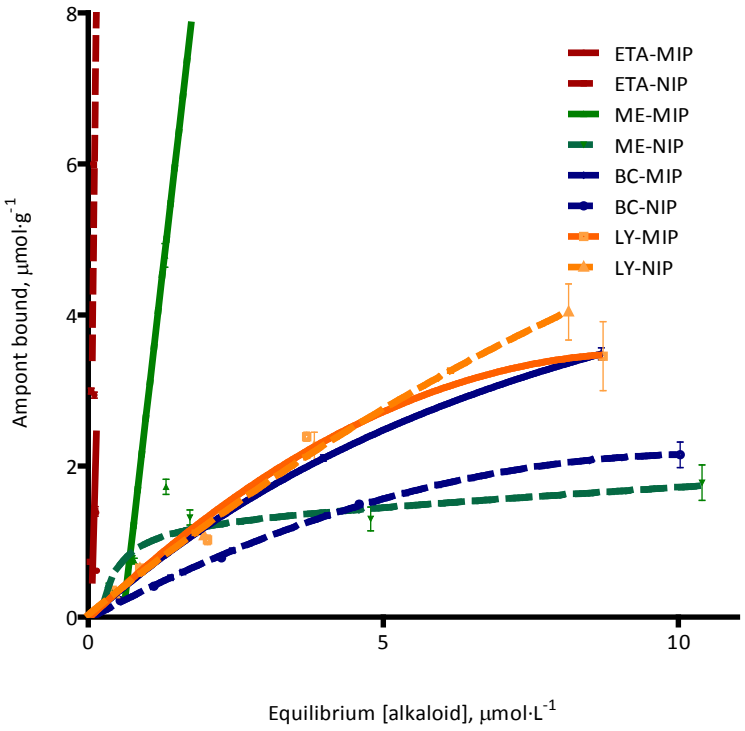


Figure 4-6. Adsorption of different types of ergot alkaloids (nM) by MIP and NIP in McDougall's buffer (pH 6.8) per mg of polymer used.

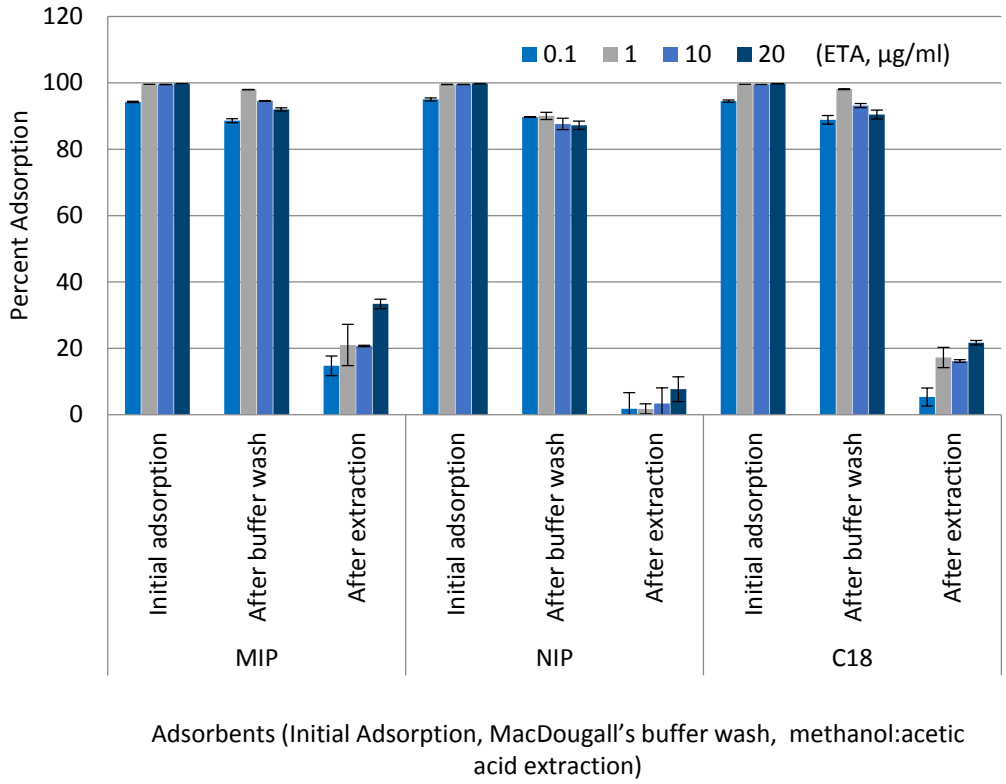


Figure 4-7. Percent retention of ETA by 100mg of MIP, NIP or C18 using MISPE method in McDougall's buffer of pH 6.8. No difference ($P = 0.23$) was observed between polymer type in initial adsorption. The percent retention after buffer wash was significantly lower ($P < 0.01$) for NIP compared to MIP and C_{18}, but no difference ($P = 0.32$) between MIP and C_{18}. Percent ETA remained on the NIP was significantly lower ($P < 0.03$) than the two other polymer types after extraction.

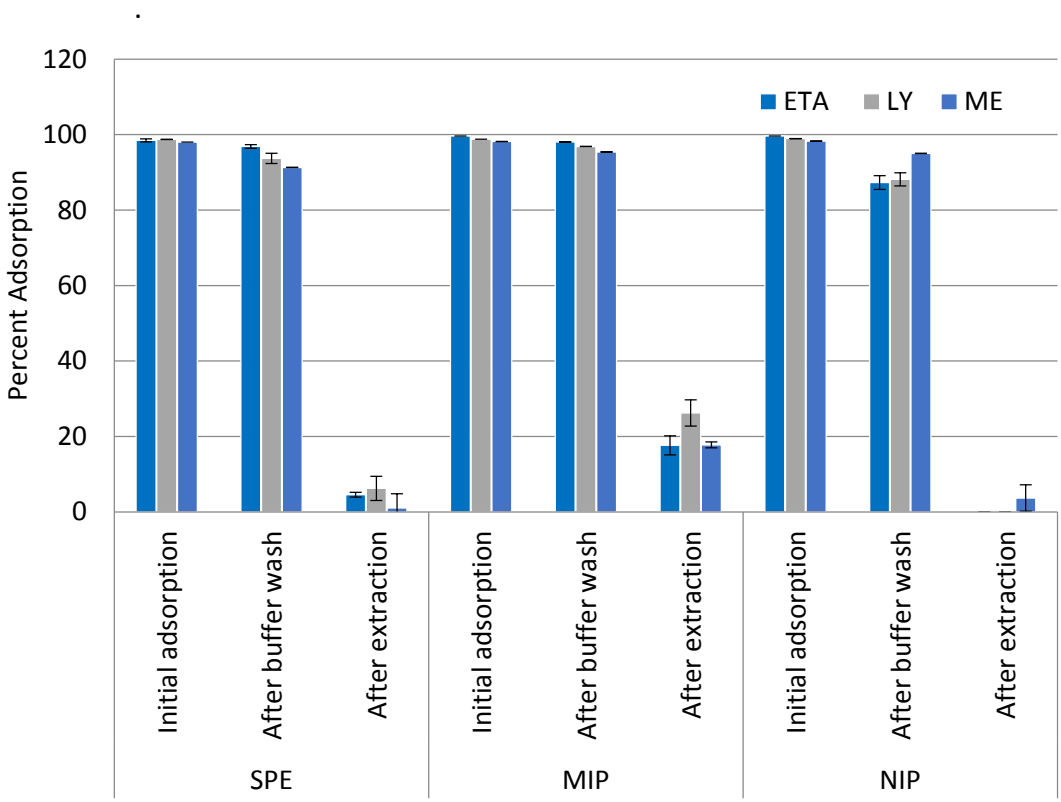


Figure 4-8. Percent retention of ETA, LY and ME by 100mg of MIP, NIP or C18 using MISPE method in McDougall's buffer of pH 6.8. There was no difference ($P = 0.40$) between products in initial retention of alkaloids. After buffer wash, MIP and C_{18} had higher ($P < 0.01$) retention of ETA and LY compared to NIP. MIP had higher retention ($P < 0.01$) of alkaloids after extraction solvent compared to NIP and C_{18}.

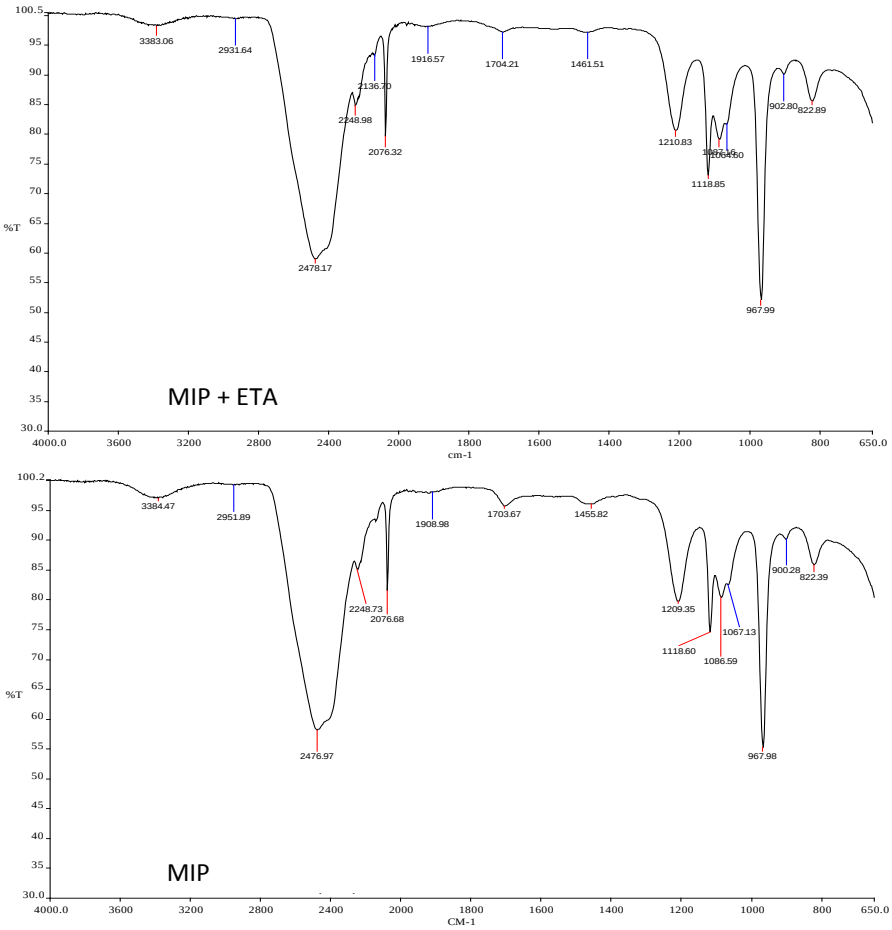


Figure 4-9. FTIR spectra of before (bottom) and after (top) exposure of the MIP to ETA in MacDougall's buffer environment (pH 6.8).

5. EFFECT OF AN IMPRINTED-POLYMER ERGOT-ALKALOID ADSORBENT ON *IN VITRO* RUMINAL FERMENTATION

5.1. Introduction

A symbiotic relation exist between the endophytic fungus (*Epichloë coenophiala)* and tall fescue (*Lolium arundinaceum)*.The fungus provides the grass resistance to environmental stress (West, 1994), insect infestation and herbivory (Elmi et al., 2000), and enhanced photosynthesis (Richardson et al., 1993), while the plant provides the fungus with nutrition, means of dispersal and protection from the environment (Hoveland, 1993). Fungal effects on the plant are partly due to production of heterocyclic compounds called ergot alkaloids as a secondary metabolite, and these have been shown to be toxic to grazing animals (Stuedemann and Hoveland, 1988). Even though ruminants are considered less susceptible than non-ruminants to toxic substances because of the detoxifying potential of rumen microbes (Gallo and Masoero, 2010), symptoms of alkaloid toxicity including elevated body temperature, decreased feed intake, excessive salivation, increased respiration rate and decreased reproductive efficiency have been well documented (Hemken et al., 1979; Schmidt et al., 1982; Evans et al., 1988; Strickland et al., 1993).

The most common mitigation techniques involve dilution with non-contaminated forage or supplements to decrease the concentration of alkaloids. Additionally, novel endophyte strains that do not produce toxic alkaloids have been created by manipulation of endophyte genes responsible for alkaloid synthesis followed by insertion into tall fescue varieties (Bouton et al., 2002). Other research has focused on use of adsorbents, like yeast cell wall preparations and clay, that would reduce the bioavailability of toxins in the GIT. Studies using yeast cell wall preparations have shown reduced bioavailability of ergotamine but little evidence of beneficial effects on animal performance (Akay et al., 2004; Aaron et al., 2006). *In vitro* studies with organoclay compounds indicated a low propensity to bind ergotamine in acidic media (Huebner, 1999).

While there exist a number of challenges towards developing effective treatment strategies, one potential method to overcome the problems associated with ergot toxicity is the addition of molecularly imprinted polymers (MIP) to the diet. Molecularly imprinted polymers are macromolecules synthesized from functional monomers and crosslinkers in the presence of template (adsorbate). Functional monomers and crosslinkers are allowed to interact with the template during polymerization and later the template is removed with appropriate solvent leaving behind accessible binding sites that are

complementary in shape and functionality to the template (Sellergren and Hall, 2001; Urraca et al., 2007; Alsudir and Lai, 2012). These MIP are stable at low and high pH, pressure and temperature (Tiwari and Uzun, 2016), and have been used where high selectivity and specificity in binding is required. Our previous studies using methacrylate based imprinted polymers have shown high adsorption efficiency of both imprinted and non-imprinted polymers (NIP) towards ergotamine tartrate in comparison to yeast cell wall preparations (Kudupoje et al., 2015).

Prior to *in vivo* evaluation, it is useful to ensure that potential dietary supplements for ruminants do not interfere with ruminal microbial function. Therefore, this study was designed to determine the effects of increasing levels of both MIP and NIP on ruminal microbial fermentation in a forage-based diet using an *in vitro* batch culture fermentation system.

5.2. Materials and methods

All animal-related procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

5.2.1. Chemicals and solvents

Chemicals and solvents used were ACS grade or equivalent. Water was ultra-pure and de-ionized through a Milli-Q treatment system (18MΩ·cm resistance, Millipore, Billerica, MA). Methanol, acetonitrile and formic acid used for liquid chromatography were HPLC grade (VWR, Suwanee, GA). Methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA, ≥97%), 1,1,3,3-tetramethylguanidine (TMG, >97%), and 2-hydroxyethyl methacrylate (HEMA, >98%) were obtained from Thermo-Fisher Scientific (Waltham, MA). 2,2-azobisisobutyronitrile (AIBN, ≥98%), 2-ethlybutyrate, phosphoric acid, metaphosphoric acid, acetate, butyrate, propionate, isobutyrate, isovalerate, valerate and ergotamine tartrate sodium salt (ETA) were purchased in purified crystalline form (≥ 97%) from Sigma-Aldrich (St. Louis, MO).

All chemicals (> 97% purity) required for buffer preparations including trypitcase-A, sodium sulfide nano-hydrate, cysteine HCl, resazurin, ammonium bicarbonate, sodium bicarbonate, calcium chloride dihydrate, manganese chloride tetrahydrate, cobalt(II) chloride hexahydrate, ferric chloride hexahydrate, sodium phosphate dibasic, potassium dihydrogen phosphate, magnesium sulfate monohydrate, sodium hydroxide and sodium sulfide nanohydrate were purchased from Sigma-Aldrich (St. Louis, MO).

5.2.2. Polymer synthesis

In our previous study, ergotamine templated methacrylic acid (MAA) based polymer was synthesized using bulk polymerization (Arshady and Mosbach, 1981). The chemicals and their proportions and preparation conditions have been described previously (Kudupoje et al., 2015a). Isothermal adsorption studies in McDougall's buffer of pH 6.8 (Kudupoje et al., 2015a) were used to determine the adsorption properties of polymers.

5.2.3. *In vitro* fermentation study

Ruminal fluid for inoculum was pooled from 4 ruminally cannulated steers (500-550 kg BW) grazing endophyte-free fescue (*Lolium arundinaceum;* KY 32, Turner Seed Co., Winchester, KY). On collection days, steers were gathered from pasture at approximately 07:30 and whole ruminal contents were collected. Ruminal contents were collected into a preheated, insulated container, transported to the laboratory and pulverized using an immersion blender for two min under a CO_2 headspace. The inoculum source for the experiment was prepared by straining the blended contents through 4 layers of cheesecloth.

Several *in vitro* gas production experiments were carried out to validate the experimental conditions necessary to determine the effect of polymers on fermentation. While conducting the first experiment using an established protocol for *in vitro* fermentation (Tilley and Terry, 1963), the control treatments (polymers without inoculum: i.e., without fermentation) produced gas, indicating some reaction between the buffer or substrate and the polymer. To evaluate buffering agents, an experiment was designed to evaluate replacing sodium bicarbonate in MacDougall's buffer with sodium phosphate dibasic. The pH and molar concentrations of the phosphate buffer remained the same as for the bicarbonate buffer. In a subsequent validation study, the amount of substrate (300 vs 500 mg DM basis) necessary to generate gas production curves with a reasonable degree of resolution was examined. These preliminary experiments were conducted using the protocol described below and the plateau in gas production was used as the criteria for choosing the best experimental condition.

In vitro gas production was determined in triplicate runs conducted on 3 separate days, with each run consisting of 3 vessels/treatment (n = 9/treatment) with a 2 x 4 + 1 factorial treatment structure. MacDougall's buffer containing micro- and macro-mineral solutions, and reducing solutions was prepared as described previously (Goering and Van Soest, 1970) with the substitution of phosphate for bicarbonate as described above A mixture of buffer (1475 mL) and of rumen inoculum (350 mL) was

prepared and placed in a water bath maintained at 39 °C and purged with CO_2 until added to the 250 mL fermentation vessels containing the treatment (polymer: MIP or NIP, 5 levels: 0, 0.3, 3, 30, 300 mg) and diet substrate (500 mg alfalfa hay DM; Table 5-1) and 2 mL of H_2O (to wet the polymer and substrate). The vessels were gassed with CO_2 for 30 seconds and then fitted with remote automatic pressure transducers (AnkomRF Wireless Gas Production System, Ankom Technology, Macedon, NY) and incubated in a water bath (39 ˚C, 30 h). Vessels were on an oscillating tray set at a speed of 50 rpm to continuously mix the ruminal culture in each vessel. The gas pressure was measured at 1 min intervals.

At the completion of the 30 h fermentation, vessels were placed into an ice bath to cease fermentation. Headspace gas samples were drawn into 10 mL vacutainer tubes (without additive) for methane analysis using gas chromatography. Ruminal culture pH was measured using a portable pH meter (Acorn pH 6 Meter, Oakton Instruments, Vernon Hills, IL). For volatile fatty acid (VFA) analysis, a 5 mL aliquot of culture fluid was added to 15 mL screw-cap polypropylene centrifuge tubes containing 0.5 mL of metaphosphoric acid (25 g/100 mL) and 0.5 mL of VFA internal standard (1 g/100 mL 2-ethlybutyrate) and stored in a freezer (-20 °C) overnight. For ammonia analysis, a 100 µL sample was combined with 3.9 mL phosphoric acid (25 mM H_3PO_4) and stored at -20 °C until analyzed. The samples were thawed before GC analysis (VFA) or colorimetric analysis (ammonia). VFA concentrations were determined using a gas chromatograph (6890 Hewlett-Packard, Avondale, PA), equipped with flame ionization detector and a split injector. Analysis was performed with a fused silica capillary column (15m x 0.53mm x 0.05 µm film thickness; Nukol™ Supelco, Bellefonte, PA) following procedures described previously (Cottyn and Boucque, 1968) The injection volume was 1µL. High purity helium was used as the carrier gas with a flow rate of 1.3 $mL \cdot min^{-1}$. Peak identification was performed using external standard. Ammonia-N was determined using a photometric test with enzymatic assay through Konelab analysis (Model 20XTi, Thermo Fisher Scientific, Waltham, MA) following procedures described earlier (Kun and Kearney, 1974).

5.2.4. Gas production measurement and modelling

The Ankom system was used to measures gas production as a change in pressure (in units of psi). Gas pressures were converted to gas volumes (mL) at standard temperature and pressure based on the ideal gas law (pV=nRT). The measurements were made at 39 °C, and the volume was calculated using the formula.

$$V_2 = \frac{p_1 \times V_1}{p_2} \times \frac{T_2}{T_1}$$

Where: V_2 = volume (in mL) at standard temperature and pressure

p_1 = pressure (in psi) as measured

V_1 = volume (in mL) of headspace of individual fermentation vessel

p_2 = pressure at standard conditions (14.6959 psi)

T_2 = temperature (in K) at standard conditions (273 K)

T_1 = temperature (in K) at measurement conditions (312 K)

Gas volumes thus measured over time were fitted with various nonlinear mathematical models to determine the kinetic parameters. To identify the best model, ten gas production models described by Pitt et al., (1999); exponential, logistic, France, Cauchy, Weibull, Gompertz, Richards, Feller, Cone and Fitzhugh) were fitted to the data and the model with the best R^2 and lowest root mean squared prediction error (RMSPE) was selected to describe the kinetic parameters.

5.2.5. Methane

Gas samples were analyzed for methane concentration by gas chromatography (6890 Hewlett-Packard, Avondale, PA), fitted with a Supelco stainless steel 40/60 carboxen 1000 packed column (1.524 m x 3.175 mm x 2.1 mm). Column head pressure was set at 0.680 Atm and oven temperature set point was 125°C (maximum 190°C) (Xu et al., 2010).

5.2.6. Sampling of fescue grass for alkaloid analysis

Confirmation of the alkaloid status of the endophyte-free pastures was achieved by analyzing clipped grass samples. Ten random 0.09 m^2 samples were obtained by cutting grass at ground level. The grass samples were chopped into smaller pieces (1 inch length) and freeze dried. The dried samples were then pulverized in coffee grinder and 10 g of sample was extracted with 25 mL of 80% ethanol for 4 h (Lehner et al., 2004; Ji et al., 2014). Ethanolic extract was applied to reverse phase C_{18} SPE columns for sample clean up (Lehner et al., 2004) and the eluent was analyzed for alkaloid concentrations by LC-MS/MS (Jackson et al., 2012).

5.2.7. LC-MS/MS analysis

The LC- MS/MS system consisted of a LC and a triple quadrupole mass spectrometer (Acquity, Waters Corp., Milford, MA) operated in positive electrospray ionization mode, with gradient mobile phase. Ergotamine (10 µl) was separated on an Acquity ethylene-bridged C_{18} hybrid column (40 °C, 1.7 µm, 2.1 mm x 100 mm, Waters Corp) with a mobile phase gradient of methanol-water (both acidified with 0.1% formic acid) from 5:95 (v/v) to 10:90 (v/v) for 2 min, then to 75:25 (v/v) for 8 min, then maintained at isocratic with 99:1 (v/v) for 2 min and finally re-equilibrated with 5:95 (v/v) for 2 min; the flow rate was 0.42 $mL \cdot min^{-1}$. Nitrogen (Nitroflow, Parker-Balston, Haverhill, MA, USA) was used as desolvation and cone gas. Standards prepared in methanol: water (50:50, v/v) were infused with UPLC flow rate of 0.42 $mL \cdot min^{-1}$ to obtain tuning parameters for analyte transitions using the Intellistart program (Waters Corp.)

5.2.8. Statistical analysis

Data were analyzed using a model appropriate for a completely randomized design with a 2 x 4 + 1 factorial arrangement of treatments using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). The model included treatments (polymer type, inclusion amount) and their interactions as fixed effects. Orthogonal polynomial contrasts were used to evaluate effects of polymer inclusion level using the contrast option with contrast coefficients appropriate for unequally spaced treatments generated by the IML procedure of SAS. Significant differences between polymer types were declared at $P < 0.05$.

5.3. Results

5.3.1. Validation studies

Initial evaluations indicated that the total gas production increased with an increase in the amount of polymers in the absence of inoculum or substrate indicating in interaction between the polymers and buffering compounds (possibly between -COOH or –OH of polymer and bicarbonate, producing CO_2). Thus, sodium bicarbonate (0.133 M) in MacDougall's buffer was replaced with an equal molar concentration of sodium phosphate dibasic and additional experiments were conducted to validate MacDougall's buffer prepared from phosphate buffer. Additionally, the substrate inclusion rate in MacDougall's buffer prepared from phosphate buffer was also validated.

Cumulative gas production with two levels of substrate (500 vs 300 mg DM alfalfa) and in two different buffering agent (MacDougall's buffer of pH 6.8, sodium bicarbonate vs sodium phosphate) is

shown in Figure 5-1. The use of 500 mg substrate provided cumulative gas pressures that were within the upper limits of the system and which provided a greater degree of discrimination for treatment effects compared with the use of 300 mg substrate. Additionally, there was no significant difference in cumulative gas production (P >0.05) between bicarbonate and phosphate buffer at substrate level of 500 mg alfalfa (DM basis). However, at 330 mg substrate level, there was a time by buffer type interaction ($p<0.05$). Therefore, 500 mg substrate (alfalfa, DM) with phosphate as buffering agent was chosen for all subsequent studies.

5.3.2. MIP synthesis and evaluation

Molar ratios between the template and functional monomers is one factor influencing binding selectivities of synthetic polymers. Methacrylate-based imprinted polymers have shown better selectivity for aromatic template when the ratios of template:monomer:crosslinkers were 0.4: 1.4: 5.6 (Nantasenamat et al., 2006). Additionally, in another study, a ratio of 1:4 for metergoline: total functional monomers showed better binding properties and selectivity towards ergot alkaloids (Lenain et al., 2012). In our study, the molar ratio of 0.06: 1.5: 5 (template: monomer: crosslinkers) was used. Isothermal adsorption studies using these imprinted polymers towards ergot alkaloids have shown high adsorption of ergotamine tartrate followed by bromocriptine, lysergol and methylergonovineKudupoje et al., 2015). These polymers were tested in MacDougall's buffer of pH 6.8, at a very low inclusion rate of 0.01% w/v relative to other adsorbents (0.1 to 2% w/v) that have been targeted towards mycotoxins (Huwig et al., 2001; Freimund et al., 2003; Sabater-Vilar et al., 2007). Based on the > 90% adsorption efficiency of MIP towards ETA (Kudupoje et al., 2015b) and typical natural contamination level of ergopeptide alkaloids of 0.5 ppm in diet (Rottinghaus et al., 1991), the inclusion rate of 0.03% w/w of MIP in the diet (as fed basis) was estimated to result in a reduction in ergotamine bioavailability to < 0.05 ppm in diet. Therefore, the calculated polymers level of 0.03 ± two log scale (0, 0.003, 0.03, 0.3 and 3%, w/v of rumen fluid) were tested in the present study.

5.3.3. Fescue grass contamination

No ergot alkaloids were detected in fescue grass sample or in the alfalfa substrate used in the *in vitro* gas production experiment.

5.3.4. IVF parameters measured

5.3.4.1. Gas production

Of the ten models evaluated, the exponential model had the best fit. The exponential model contains 2 parameters, V_{max} and r:

$$V_t = V_{max}\ (1 - e^{-rt})$$

where:

V_t = volume of gas accumulated (ml) at time (t)

V_{Max} = Maximum volume of gas produced, mL

r = rate of gas production, 1/h

t = time, h

The fitted gas production kinetic parameters after 30 h of incubation of alfalfa substrate (500 mg DM) with increasing levels of polymer are detailed in table 5-2. There were no differences (P = 0.31) between polymer types and no interactions (P > 0.69) between polymer type and inclusion level for maximum gas production and rate of gas production. Additionally, increasing polymer concentrations did not influence the rate of gas production (P > 0.32) (Figure 5-2; Table 5-2) or total gas production (P > 0.32).

5.3.4.2. pH

There was no interaction (P = 0.81) between the polymer type and inclusion rate. There was no difference between the polymer type (P = 0.24). However, the average mean of inclusion decreased (P <0.001) the pH linearly from 6.49 to 6.30 as the polymer concentration in 100 mL diluted rumen fluid containing 500 mg of alfalfa substrate (DM) increased from 0 to 300 mg (Figure 5-4; Table 5-3). Methane

The average CH_4 produced as a percent of total gas was 12.55% (Table 5-3), and there was no difference between the polymer type (P = 0.53) or interaction between polymer type and inclusion rates of polymer (P = 0.08). Additionally, % CH_4 produced was not affected (P >0.17) by inclusion rates of polymer.

5.3.4.3. VFA

Concentrations of VFA after 30 h of incubation and molar proportions of individual VFA are detailed in table 5-3. No polymer concentration effects were observed for individual VFAs (P >0.10) or for total VFA. However, increasing the polymer concentrations had a tendency to affect acetate and total VFAs in cubic fashion (P =0.08) but did not affect (P > 0.10) other VFA concentrations. There was no difference between polymer types (P > 0.35) and no interactions (P >0.49) between polymer type and inclusion level.

5.3.4.4. NH3

For ammonia-N concentrations (Table 5-3) there was no difference (P =0.86) between the MIP and NIP and also there was no interactions (P =0.69) between the polymer type and inclusion rates.

5.4. Discussion

Generally, the rate of gas production is positively correlated with rate of substrate disappearance. Carbon dioxide acts as a Lewis acid in the presence of Bronsted and Lewis bases (Hildebrand et al., 1970; Reilly et al., 1995). Polymers possessing electron-donating functional groups (e.g., carbonyl groups) exhibit specific interactions with CO_2, because of Lewis acid−base nature. Research has shown the potential of weak acid base interaction between CO_2 and the C=O oxygen of PMMA (Kazarian et al., 1996). However, increasing level of polymer from 0 to 300 mg in 100 mL diluted rumen fluid containing 500 mg of alfalfa substrate (DM) had no effect (P >0.32) on the rate of gas production.

Methane from enteric fermentation is a natural by-product of the digestive process of ruminants and it represents a loss of 2 to 12% of the animal's gross energy intake (Johnson and Johnson, 1995; McAllister et al., 1996). Methane is synthesized by methanogenic archaea using mainly CO_2 and dihydrogen (H_2) that are produced by ruminal anaerobic fermentation of carbohydrates (Moss et al., 2000; Wilkinson, 2012). *In vitro* experiments have shown that polyphenolic compounds (tannins) and polymers of flavan-3-ol units (proanthocyanidins) can bind to fibers and decrease their degradability by ruminal microorganisms, thus diminishing the production of H_2 and therefore hampering ruminal methanogenesis (Theodoridou et al., 2011; Jayanegara et al., 2015). Both polymers (tannins and proanthocyanidins) have been hypothesized to inhibit the activities of methanogens directly and specifically. A study of the structure-activity relationship of oligomeric ellagitannins on ruminal

fermentation *in vitro* has shown that large oligomers have more detrimental effects on volatile fatty acid and gas production than small ones, and are similarly effective at inhibiting methane production (Baert et al., 2016). Additionally, polyethylene glycol (PEG), a polymer that is used as a tannin complexing agent, has been shown to increase *in vitro* gas production, ammonia N concentration and short chain fatty acid production (Khazaal et al., 1996; Getachew et al., 2000). In the present study, polymers prepared using methacrylate monomers that were crosslinked with ethylene glycol dimethacrylate did not influence methane production or VFA profiles indicating both polymers were essentially inert at inclusion rates between 0 and 300 mg per 0.5 g of DM.

The pH of ruminal contents has a significant impact on microbial activity, rumen function, and animal productivity and health (Nagaraja and Lechtenberg, 2007). Ruminal acidosis is a fermentation disorder in the rumen characterized by a lower than normal ruminal pH, that reflects an imbalance between microbial production, microbial utilization, and ruminal absorption of VFAs (Castillo et al., 2012). It can change fiber digestion by modulating microbial attachment to fiber particles (Sung et al., 2007). Fibrolytic bacteria are very sensitive to ruminal pH, with optimum functioning pH level between 6 and 7 (Russell and Wilson, 1996). Additionally, adhesion of *R. albus* (fibrolytic bacteria) was decreased markedly when pH level dropped below 5.0 (Morris, 1988). In the present study, the pH values decreased as the polymer level increased in the media. One could expect that VFA concentrations in the rumen fluid are the main source of H+ that influence the pH change. However, there was no difference (P >0.5) in VFA and NH_3 profile between the treatments suggesting direct effects of polymer on pH. The pKa of polymers made from acrylates including poly-methylacrylic acid (Kim and Peppas, 2002) and poly-acrylic acid (Yang et al., 2004) were shown to be nearly 5 and 4.28, respectively. The carboxyl group of PMAA would be ionized at the pH of this study (6.8), which could be the primary contributing factor to the observed marginal decrease in pH. However, the pH was within the normal range (6.19 – 6.5) for optimum microbial function.

In general, on forage-based diets, the ammonia level in the rumen fluid depends on time of sampling post feeding, diet composition, intake level and microbial growth and activity. Ammonia nitrogen usually ranges between 20 $mg \cdot dL^{-1}$ pre-feeding to 40 $mg \cdot dL^{-1}$ post feeding as a results of protein degradation and amino acid deamination. (Adams and Kartchner, 1984). Ammonia nitrogen in the rumen must be above a critical level (20 $mg \cdot dL^{-1}$) for a considerable period of the day to ensure a high rate of microbial growth (Leng, 1991). Incubating alfalfa substrate (500 mg, DM) in diluted rumen fluid for 30 h, at pH 6.8, had ammonia nitrogen concentrations of approximately 17 $mg \cdot dL^{-1}$ (10mM). The

results indicated that there was no difference between the control (0 mg polymer) and the treatments containing polymer suggesting that protein degradation and amino acid deamination profile were not affected by the treatments, and similarly that NH_3 availability and uptake were unaffected by polymer inclusion.

For the intended application these polymers need to be tolerant to ruminal fermentation end products, along with mild variation in pH and temperature. Polymers intended for biomedical applications have used biodegradable polymers synthesized using methacrylate monomers that can be hydrolyzed and are also water soluble in a non-cross liked form. Polymers synthesized using polyethylene glycol methacrylate (PEGMA) monomers and poly(lactide-co-glycolide)-polyethylene glycol (PGLA-PEG) crosslinkers using a suspension polymerization are shown to undergo hydrolytic degradation to low-molecular-weight compounds (PEG, lactic and glycolic acids) and water-soluble polymethacrylate chains in PBS at 37 °C in less than 2 days (Louguet et al., 2014). Additionally, the same polymer was shown to rapidly degrade when implanted subcutaneously in rabbits and caused a mild and transient inflammatory reaction. In the event of those polymers being susceptible to hydrolysis, mainly monomeric units would have formed. Even though functional monomers like ethyl acrylate and methyl methacrylate are known to depress feed intake in rats at > 2000 ppm in the diet, no mortality or hematological and histopathological lesions were observed (Borzelleca et al., 1964). Additionally, structural requirements for acrylate esters to cause gastric toxicity include the ester moiety, a double bond and no substitution at carbon number 2 (Ghanayem et al., 1985). However, polymer degradation is less likely in the rumen environment. Poly-methacrylic acid is stable up to about 200 °C (Grant and Grassie, 1960) and salts of poly acrylic acid are stable up to 400 °C (McNeill and Sadeghi, 1990). Poly acrylic acid-based materials are highly stable within weak acid and weak basic pH conditions and moderate ionic strengths in buffer solution (Liu et al., 2006). Additionally, the utility of polymers made of styrene, isoprene, butadiene, and an alkyl acrylate as coating materials for nutrients that need to bypass the rumen has been patented (Dannelly and Ardell, 1980). In the present study, polymer inclusion at levels between 0.03 to 30 mg per 500 mg substrate, for 30 hours at pH of 6.8, did not affect microbial fermentation suggesting their inert nature with respect to the rumen environmental conditions.

Another concern with the addition of adsorbents is potential non-specific binding to feed nutrients. Nutrient deficiencies can result in low microbial yield relative to VFA and also lead to a low protein (from microbes) to energy (from VFA) ratio in the nutrients absorbed. Even though nonspecific

binding of polymer to ruminal nutrients was not directly assessed in this study, fermentation characteristics were largely unaffected by polymer inclusion, suggesting similar availability of nutrients necessary for microbial growth.

5.5. Conclusion

While there is a number of challenges towards developing effective mitigation strategies to reduce ergot toxicity, the present s could provide effective alleviation strategy for prevention of alkaloid toxicity. Our previous studies have shown that MAA based polymers synthesized in the lab had superior adsorption properties compared to styrene or clay based polymers. Studies have shown toxicity of functional monomers like ethyl acrylate and methyl methacrylate in their monomeric form, but not in polymeric form. Although polymer degradation is unlikely in the rumen environment, their effect on ruminal fermentation in their polymeric form was also of interest. This study showed that polymer exposure at levels between 0.03 to 30mg per 500mg alfalfa DM, for 30 h at pH of 6.8, did not affect microbial fermentation. At very high levels (300 mg), small effects on fermentation pH were noted. At levels which would be reasonable to include in ruminant rations (between the ranges of 0.03 to 0.3 g· kg^{-1} that provides adsorption of more than 90 percent), these polymers had no detectable effect on *in vitro* fermentation suggesting absence of nonspecific binding to nutrients required for microbial growth. While these polymers are serving a very important purpose in analytical chemistry as solid phase extraction material, or as slow drug delivery system in medical field, the adsorption properties and their inert nature gives them also an advantage if used as adsorbent material in feeding strategies for production animals to mitigate the ergot toxicity. Even though the *in vitro* fermentation experiments mimic only a fraction of the complex interactions during rumen fermentation, they still fall short at accounting for the complete dynamics of the GIT. Of specific interest, since ergot alkaloids have been characterized as vasoconstrictors, and *ex vivo* models using veins contractibility could be utilized to established the incidence of ergot alkaloids and the impact of a polymeric material as a mitigation strategy.

Table 5-1. Nutrient composition of alfalfa substrate.

Nutrient, DM basis[a]	%, DM basis
Crude protein, %	17.1
Acid Detergent Fiber, %	39.5
Neutral Detergent Fiber, %	47.8
NFC, %	21.1
Calcium, %	2.4
Phosphorus, %	0.20
Magnesium, %	0.36
Potassium, %	1.43
Sodium, %	0.159
Sulfur, %	0.35
Iron, ppm	576.0
Zinc, ppm	15.0
Copper, ppm	10.0
Manganese, ppm	35.0
Molybdenum, ppm	1.10
NE_m, Mcal/kg	1.28
NE_g, Mcal/kg	0.71

[a]Based on analysis by Dairy One, Ithaca, NY.

Table 5-2. Effect of methacrylate based synthetic polymer on gas production parameters from *in vitro* fermentation of 0.5g of alfalfa substrate (DM) with rumen inoculum during 30 h incubation period.

	Polymer inclusion level , mg per 500 mg alfalfa (DM)									SEM	P-Value				
Parameters	CONT	MIP				NIP					Prod x Level	Prod	Level		
	0	0.3	3	30	300	0.3	3	30	300						
V_{Max} [a]	81.67	77.57	75.27	74.46	75.40	77.13	78.11	79.04	77.28	3.071	0.87	0.32	0.44	0.47	0.32
k [b]	0.13524	0.1332	0.1404	0.1356	0.1386	0.1362	0.1344	0.1314	0.132	0.0048	0.69	0.32	0.83	0.81	0.33

[a] maximum volume of gas produced (mL)

[b] rate of gas production, $1 \cdot h^{-1}$

Table 5-3. The effect of polymers on *in vitro* fermentative end products of alfalfa substrate (500 mg DM).

	Polymer inclusion level, mg per 500 mg alfalfa (DM)									SEM	P-Value				
Parameters	Control	MIP				NIP					Polymer x Level	Polymer type	Level		
	0	0.3	3	30	300	0.3	3	30	300				Linear	Quadratic	Cubic
Ammonia nitrogen, mM/L	9.59	10.10	9.77	9.66	9.85	9.73	9.85	9.56	9.60	0.19	0.69	0.86	0.87	0.31	0.56
CH_4 (% of total gas)	12.65	12.55	12.71	12.51	12.70	12.18	12.78	12.87	12.01	0.22	0.08	0.53	0.17	0.42	0.33
pH	6.49	6.49	6.50	6.46	6.30	6.48	6.47	6.42	6.19	0.04	0.0002	0.88	<.0001	0.60	0.86
Total VFA, mM	50.35	45.77	42.97	41.66	47.05	46.21	42.27	45.17	47.21	3.42	0.72	0.90	0.64	0.19	0.08
VFA															
Acetate, mM	31.58	28.39	26.35	25.47	29.56	28.48	25.81	27.81	29.38	2.48	0.74	0.90	0.57	0.20	0.08
Propionate, mM	8.10	7.41	6.95	6.57	7.44	7.47	6.76	7.20	7.72	0.60	0.75	0.89	0.67	0.16	0.10
Butyrate, mM	5.19	4.99	4.81	4.59	4.69	5.18	4.80	5.04	5.10	0.30	0.83	0.83	0.68	0.36	0.26
Isobutyrate, mM	1.83	1.16	1.13	1.26	1.89	1.17	1.10	1.18	1.17	0.26	0.17	0.10	0.41	0.36	0.11
Isovalerate, mM	1.93	2.07	2.02	2.04	1.85	2.10	2.04	2.13	2.09	0.12	0.70	0.58	0.54	0.45	0.82
Valerate, mM	1.71	1.74	1.71	1.72	1.61	1.80	1.74	1.80	1.73	0.07	0.70	0.52	0.29	0.58	0.90
A:P	3.9	3.8	3.8	3.9	4.0	3.8	3.8	3.9	3.8	0.06	0.49	0.35	0.38	0.78	0.23

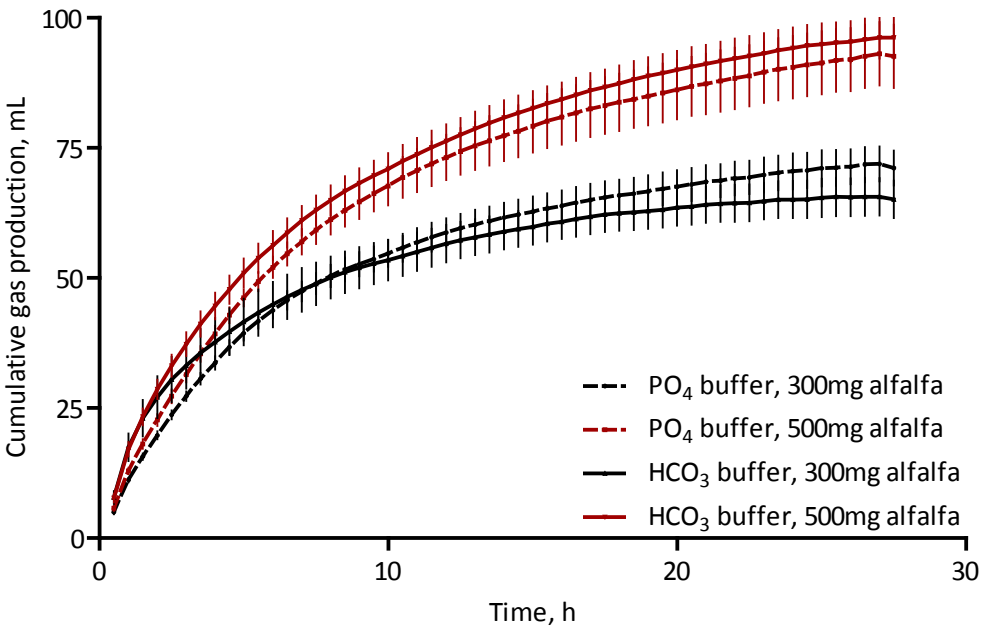


Figure 5-1. Total cumulative gas production in MacDougall's buffer of pH 6.8 prepared either with sodium bicarbonate or potassium phosphate, at two levels of substrate (DM basis). (Red: 500 mg substrate, black: 300mg substrate; solid line: HCO_3 buffer, dashed line: PO_4 buffer, vertical lines are SEM (n=8))

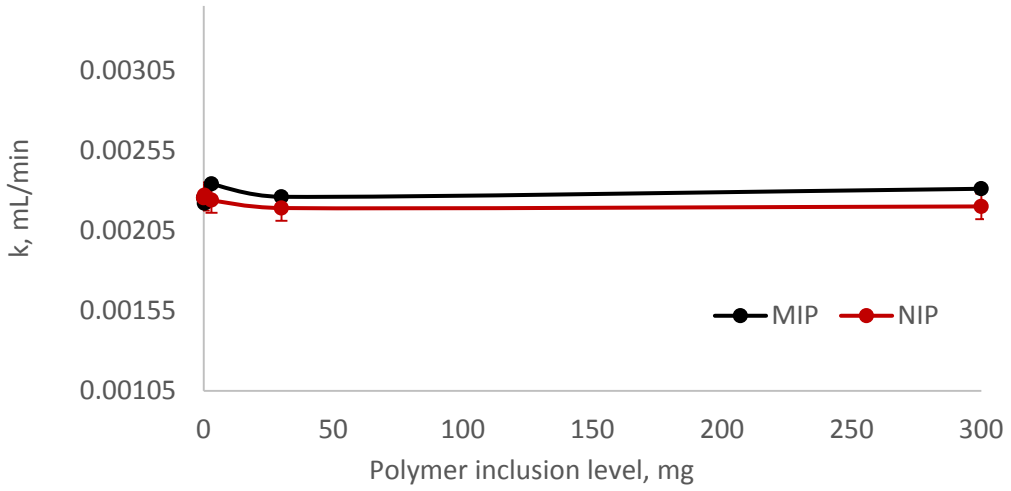


Figure 5-2. Effect of polymer inclusion level on rate of *in vitro* fermentative gas production (0.5 g alfalfa substrate). There was no difference (P >0.05) between polymer type at each inclusion level and no difference (P >0.37) between the means of polymer type.

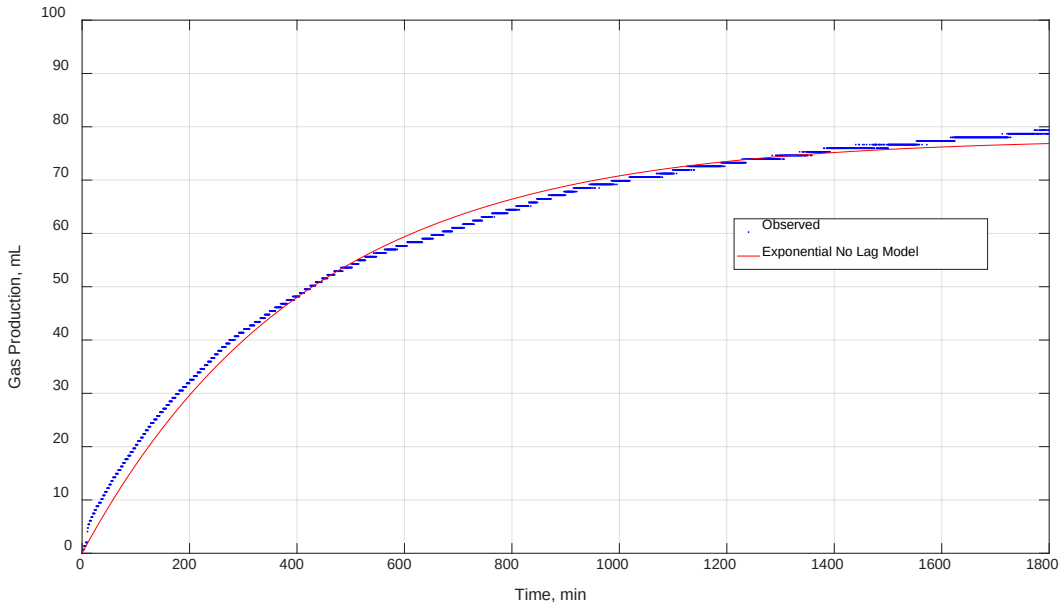


Figure 5-3. Representative example of exponential model for cumulative gas production (Polymer inclusion was 3 mg, 0.5g alfalfa substrate).

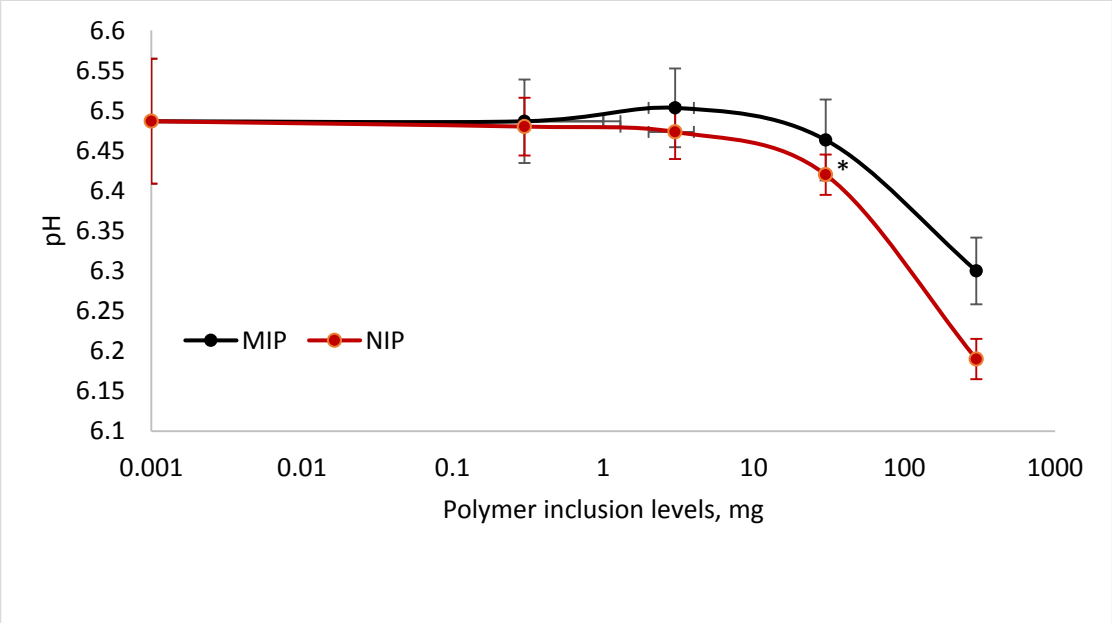


Figure 5-4. Effect of polymer on pH of ruminal fluid. There was no interaction (*P* = 0.81) between the polymer type and inclusion rate. There was no difference between the polymer type (P = 0.24). The mean inclusion for both polymer decreased (*P* <0.001) the pH linearly.

6. CONTRACTILE RESPONSE OF BOVINE LATERAL SAPHENOUS VEIN TO ERGOTAMINE TARTRATE EXPOSED TO DIFFERENT CONCENTRATIONS OF MOLECULARLY IMPRINTED POLYMERS

6.1. Introduction

Alkaloids produced by the fungus *Epichloë coenophiala* are known to produce complex biological responses in animals due to agonistic and antagonistic interactions with biogenic amine receptors (Millan et al., 2002). Ergot alkaloids can induce strong and prolonged vasoconstriction in human coronary and temporal arteries (Østergaard et al., 1981), in canine saphenous and femoral veins (Müller-Schweinitzer, 1979), and in arterial preparations from rats and guinea pigs (Schöning et al., 2001). Myographic studies using several ergot derivatives have confirmed the vasoconstrictive effects in bovine preparations of lateral saphenous vein (Klotz et al., 2007), right ruminal artery and vein (Foote et al., 2011a), and mesenteric artery and vein (Egert et al., 2014). Studies have also established interactions between ergot alkaloids and neurotransmitter receptors, indicating the structural similarities between the alkaloids and biogenic amines (neuro-hormones) like serotonin (Spring et al., 2003; Klotz et al., 2012), norepinephrine (Innes, 1962; Williams et al., 1976) and dopamine (Ishii and Takayanagi, 1982; McLeay and Smith, 2006) suggesting the cause for vasoconstriction. Additionally, among two structural classes of ergot alkaloids, ergopeptides (e.g. - ergotamine, ergocornine, ergocryptine and ergocristine) are known to produce more persistent dose dependent vasoconstriction, while ergolines (e.g. – ergonovine and lysergic acid) produced rapid but lower intensity and less persistent contractile responses (Pesqueira et al., 2014).

Ergot alkaloids contain C9=C10 double bond that can readily epimerize with respect to the centre of symmetry at C-8, especially in the presence of alkalis forming a series of right-hand rotation (S)-isomers representing isolysergic acid derivatives (Komarova and Tolkachev, 2001). The C-8 epimers differ in biological and physicochemical properties and have different pKa values (Stoll et al., 1954; Maulding and Zoglio, 1970). Studies have shown that C-8-(R) isomers (-ines) are biologically active, while the C-8-(S) isomers (-inines) are inactive (Berde and Stürmer, 1978; Pierri et al., 1982). The conversion of -ines to -inines is rapid, especially in aqueous acidic or alkaline solutions (Komarova and Tolkachev, 2001) and the -inines are likely to be reactivated to their biologically active -ine form (Lampen and Klaffke, 2006). Studies have shown that ergopeptinines can also convert back into the -ine form, e.g. in methanol, aqueous

organic solvents and acids (Buchta and Cvak, 1999). Additionally, ergot alkaloids are sensitivity to light (Rutschmann and Stadler, 1978; Komarova and Tolkachev, 2001) and heat (Scott and Lawrence Guillaume, 1982; Bürk et al., 2006) which leads to both isomerization and degradation. Therefore, it is important to evaluate the toxic effects of ergot alkaloids on animals considering the possible epimerization.

Studies have also suggested the application of toxin adsorbents to alleviate the negative effects of endophyte toxins by reducing the bioavailability of toxins in the GIT. Esterified glucomannan, a derivative of cell wall of yeast (*S. cerevisiae*) and commercially available clay-based products have been shown to bind ergotamine *in vitro* (Evans and Dawson, 2000). Results from the same study indicated a decrease in adsorption efficiency in the presence of complex media like rumen fluid and MacDougall's buffer. A study conducted by Akay et al., (2003) demonstrated the potential of yeast cell wall derivative (FEB-200^{TM}) to decrease the bioavailability of fescue toxins by evaluating the excretion profile for ergot alkaloids. The mean fecal total ergovaline (ergovaline + ergovalinine) concentration of steers fed endophyte-infected tall fescue seed was significantly increased in the presence of FEB-200^{TM}. However, some classical signs of ergot toxicity including decreased dry matter intake and increased rectal temperature remained in the FEB-200-treated steers due incomplete removal of toxins. On the contrary, body weight was improved in steers fed endophyte-infected tall fescue supplemented with modified glucomannan compared to self-fed liquid supplement (Gunter et al., 2009) and also alleviated some symptoms (depressed prolactin levels and depressed average daily gain) of fescue toxicosis (Merrill, 2007).

The *in vitro* evaluation of clay and yeast cell wall-based adsorbents indicated dose-dependent interactions with ergotamine (Evans and Dawson, 2000). However, their adsorption specificities (maximum adsorption capacity and affinity) were lower in presence of interfering compounds. Decreased adsorption mainly occurs when the adsorbents are nonselective in binding, leading to nonspecific interactions (Whitlow, 2006). Therefore, adsorbents that have good adsorption properties that can achieve specific adsorption with superior affinity and selectivity is required.

In recent years, molecular imprinting technologies have gained importance due to specific binding through selective recognition. Molecular imprinting is a technique for

synthesizing macromolecular polymers with multi-functional receptor groups that aid in specific interaction with a targeted molecule (Karsten and Klaus, 2000; Sellergren and Andersson, 2000; Lian et al., 2015). These molecularly imprinted polymers (MIP) are synthesized using functional monomers, crosslinkers, initiator and porogen in the presence of an adsorbate of interest, called a template. The template interacts with functional monomers during polymerization, which is then networked with crosslinkers to form polymers of large molecular weight (Sellergren, 2001; Cormack and Elorza, 2004). These polymers are then washed to remove the template, thereby creating heterogeneous binding sites with functional groups arranged in geometries capable of binding the template and similar molecules with varying affinities (Umpleby et al., 2004). Because of specific molecular recognition properties, MIPs have been applied in different fields, especially as an alternative to antibodies (Haginaka, 2009), as a sorbent material for sample clean-up in analytical chemistry (Xu et al., 2011) and as sensors in biomedical applications (Malitesta et al., 2012).

To better understand how best to employ adsorbents in the mitigation of ergot alkaloid toxicity, the kinetics must be studied in a biological system. The kinetics of adsorbent-adsorbate interactions are profoundly influenced by the equilibrium conditions. The *in vitro* efficacy of adsorbents may be erroneously interpreted with respect to their physiological response, due to the simplified nature of *in vitro* experimental models, without encountering biological events like the hydrolytic enzymes, various pH levels, and presence of nutrients and complex media that could change the efficacy. Therefore, as a complement to *in vitro* studies, *ex vivo* bioassays are used as means to bridge the gap between the *in vitro* studies and *in vivo* experiments, which convey the message of the physiological implications of the *in vitro* studies. Bioassays have become an important part of research in various scientific fields, especially in molecular biology to test drugs and toxic compounds using cell culture models (Xu et al., 2017), in crop science for pesticides using a leaf-dip method (Galdino et al., 2011), in toxicology for lethal doses of mycotoxins using cytotoxicity and bacterial bioluminescence technique (Yates and Porter, 1982; Thompson and Wannemacher, 1986; Hanelt et al., 1994; Cetin and Bullerman, 2005) and in food chemistry for metabolism of nutrients and compounds using 2D cell culture (Mulac et al., 2011). The interactions between ergot alkaloids and neurotransmitters has been extensively studied using myograph-based techniques (Oliver et al., 1993; Klotz et al., 2006; Klotz et al., 2007; Foote et al., 2011a). Myographic bioassays are based on the contractile response of smooth muscle

present in blood vessels due to agonistic or antagonistic stimulation of neuro-receptors present on muscle cells. Ergotamine and ergovaline have been shown to produce a dose dependent smooth muscle contraction of blood vessels (Klotz et al., 2007).

The evolved design and chemistry behind the MIPs with interesting physiochemical characteristics has huge application in various fields. The advantages of using polymers, in terms of biological performance, as well as the challenges of using MIPs within this multifaceted physiological arena are not well understood. Generally, *in vitro* isothermal adsorption studies are used to evaluate the adsorbents for their adsorption efficiency. However, the physiological significance of these *in vitro* adsorption studies has to be established and the understanding of physiological implication of addition of adsorbents needs to be assessed. To aid understanding of the physiological significance, this research aimed at using *ex vivo* models to investigate the physiological significance of MIP and NIP application using *ex vivo* myographic bioassays and to compare the impact of MIP and NIP on ergot alkaloid bioavailability.

6.2. Materials and methods

Procedures used in this study did not require approval from the University of Kentucky (UKY) Animal Care and Use Committee because the blood vessels were collected from animals designated for slaughter in a local abattoir or the University of Kentucky Meats Laboratory.

6.2.1.Animals and tissues

Experiments were conducted using the cranial branch of the lateral saphenous vein which was dissected from both hind limbs of mixed breed and gender cattle (n = 17; BW 572 ± 96 kg; 20 -36 months), shortly after the animals were slaughtered (stunned with captive bolt and exsanguinated) at the University of Kentucky or local abattoir according to established procedures (Klotz et al., 2006). Excised tissue sections were placed in a modified Krebs-Henseleit oxygenated buffer (95% O_2 + 5% CO_2; pH = 7.4; mM composition =D-glucose, 11.1; $MgSO_4$, 1.2; KH_2PO_4, 1.2; KCl, 4.7; NaCl, 118.1; $CaCl_2$, 3.4; and $NaHCO_3$, 24.9; Sigma Chemical Co., St. Louis, MO) that was cooled on ice, and transported to the laboratory. Adipose and connective tissue surrounding the blood vessel was removed and the cleaned vessel was sliced into 2-mm cross-sections under a 12.5x magnifying dissecting microscope (Stemi 2000-C; Carl Zeiss Inc., Oberkochen, Germany), without damaging the endothelium. Before mounting the blood vessels in myograph chambers, the lumen, outer diameter and width of the sections were measured

under a light microscope to confirm structural integrity and consistent dimensions across sections (Axiovision, version 20; Carl Zeiss Inc.).

6.2.2.Myograph Experiments

Prior to mounting of blood vessels, 12 myograph chambers (MT610M, Danish Myo Technologies, Atlanta, GA.) were calibrated for tension with 2-g calibrated weights. The blood vessel segments were individually mounted on calibrated luminal supports in separate myograph chambers and equilibrated in 5-mL of modified Krebs-Henseleit (37 °C) containing desipramine (3.00E-05M; D3900 Sigma Chemical Co.) and propranolol (1.00E-06M; P0844, Sigma Chemical Co.) to inactivate neuronal uptake of catecholamines and to block β-adrenergic receptors, respectively (Klotz et al., 2006). The buffer solution was replaced at 15-min intervals throughout the equilibration period for 90 min to achieve baseline tension of 1 g. Norepinephrine (500-µL, 1.00E-04 M) was added to check the viability of the tissue and for subsequent normalization of the tissue response data. Before the additional of test samples, blood vessels were returned to baseline tension of 1 g by washing with modified Krebs-Henseleit buffer at 15-min intervals. The maximum tension observed during a 60-min incubation period after the addition of a treatment was recorded and corrected for baseline tension measured just before the addition of the treatment.

6.2.3.Dose response to Ergotamine

Dose response curves were generated by exposing blood vessels to increasing concentrations of ergotamine tartrate (ETA). A stock solution of 20 mM ETA was prepared (11.63mg/mL DMSO; MW: 581.66, ≥ 97% purity, #45510, Fluka, Sigma Chemical Co.) and aliquoted in 100-µL volumes into silanized HPLC vials and stored at -20 °C until use. A diluting buffer was prepared by adding 500 µL of DMSO to 99.5 mL of modified Krebs-Henseleit buffer. Ergotamine working solution of 1.000E-03 M was prepared by adding 100-µL volume of stock solution to 19.90 mL of diluting solvent. Subsequently, ten serial dilutions (1:1) were made using the diluting solution to yield working solutions of 5.000E-05, 2.500E-05, 1.250E-05, 6.250E-06, 3.125E-06, 1.563E-06, 7.813E-07, 3.906E-07, 1.953E-07 and 9.766E-08 M, and a control of 0.00 M ETA and 5-mL aliquot was added to the myograph chamber and incubated for 1 h at 39 °C. No buffer replacement occurred during this 1-h incubation period and each blood vessel cross-

section was only exposed to a single ETA concentration. At the end of the incubation period an aliquot of norepinephrine (500-µL, 1 x 10^{-4} M) was added to confirm tissue viability.

6.2.4.Polymer evaluation: Isothermal adsorption studies

Methacrylic acid based polymer adsorbents were synthesized according to a previously published protocol (Kudupoje et al., 2015). Briefly, suspension polymerization with self-assembly was used in the synthesis of polymers. During synthesis of molecularly imprinted polymer (MIP), non-molecularly imprinted polymer (NIP) without template was synthesized using the same protocol. Ergotamine tartrate (5.00E-03 mol), methacrylic acid (MAA) (8.00E-02 mol) and hydroxyethyl methacrylate (HEMA) (4.00E-02 mol) were dissolved in 10 mL toluene in a 250-mL round bottom flask and purged with high-purity dry nitrogen for 30 min at room temperature. Following the incubation period, ethylene glycol dimethacrylate (EGDMA) (4.00E-01 mol), 2,2-azobisisobutyronitrile (AIBN) (4.00E-03 mol) and toluene (65 mL) were added. Polymerization was initiated by placing the reaction mixture in an oil bath set at 65°C and propagated for 5h with stirring. Polymerization was terminated by reducing the temperature to 22 °C. The mixture was then filtered and the pellet was subjected to a template removal procedure using acidic methanol (0.2NHCL in methanol (100mL, 5 times), and acetonitrile (100mL, 5 times). The polymer products were freeze dried overnight and oven dried (60 °C for 24 h) before using in the study.

Isothermal adsorption studies were validated for inclusion rates of polymer that would generate a dose response curve showing affinity and saturation. Two independent studies were conducted with inclusion rates between 0.001 and 10 mg in Log_{10} scale and between 0 and 10 mg in log_2 scale. Responses from log_2 scale yielded a better curve fit and was used for all polymer evaluations with the myographic studies. Six inclusion levels of polymer were prepared by weighing 0, 0.625, 1.25, 2.5, 5 and 10 mg of MIP or NIP in 30-ml silanized amber color bottles in duplicate. An ergotamine tartrate working solution was prepared by adding 4.68 µl of stock solution into 120 ml Krebs-Henseleit incubation buffer yielding 7.813E-07M concentration. A 10-ml volume of ETA working solution (7.813E-07M) was added to each bottle containing different polymer masses and incubated for 5 min with shaking. The solution was then filtered using a solid phase extraction cartridge (SPE) to separate the polymer-ETA complex from free ETA, and a 5-mL aliquot of the filtrate was placed in a water bath for 15 min to reach temperature of 39 °C.

The treatments were added to the myographic chambers and incubated for 1h at 39 °C. At the end of the incubation period, norepinephrine (500-µL, 1 x 10^{-4} M) was added to confirm tissue viability. Each myograph chamber received only one treatment and treatment concentrations were administered in duplicate within each animal. Additionally, to determine adsorption efficiency, an aliquot of 1-ml from SPE filtrate was collected in silanized amber HPLC vial and analyzed for ETA concentrations by HPLC-fluorescence.

6.2.5.Validation studies

Ergotamine concentration of 7.81E-07 M that represented the 75th percentile contractile response from the dose response curve was chosen for all the polymer adsorption studies. The 75th percentile was chosen to ensure that the treatment ETA concentrations after the addition of polymers produces contractile response in the linear range of the sigmoidal dose response curve. Additionally, for accuracy and reliability, the isothermal adsorption conditions including incubation time (10 min vs 60 min), temperature during adsorption (39 vs 21 °C) and non-specific binding (SPE vs No-SPE) that would possibly affect myographic results were examined and validated.

6.2.6.HPLC analysis of Ergotamine

A series 2695 Alliance HPLC separation module (Waters, Milford, MI) equipped with a binary pump, an auto sampler, column oven and a fluorescence detector (474 scanning fluorescence detector, λ_{ex} 250nm, λ_{em} of 420nm, gain 16 and attenuation 1000) was used to analyze ergot alkloids in the samples. Chromatographic data were integrated using Waters Empower 3 software 7.00.00.99 (Waters, Milford, MI). HPLC separations were performed with a 100 x 4.6 mm i.d., 2.6 µm particle size, Kinetex C_{18} column (Phenomenex, Torrance, CA) with a gradient elution consisting of two mobile phases, A) water and B) acetonitrile, both spiked with ammonium hydroxide (0.04%). Initial gradient conditions were 100% A held for 1 min, increasing linearly to 100% B over 12 min and held for 3 min. Finally, a linear return to initial condition over 3 min, which was then held for 2 min for a total run time of 21 min. The sample injection volume was 50µL. Samples were evaporated to near dryness and the residues dissolved in methanol/water (50/50) and analyzed (Rottinghaus et al., 1991).

6.2.7.Data and Statistical Analyses

The myographic contractile response was recorded at the end of incubation period, as g of tension, using 16/35 Powerlab and Chart software (Version 8.1.3; AD Instruments, Colorado Springs, CO). The data were normalized to maximum contractile response of norepinephrine from the same tissue (equation 1), to minimize animal to animal variations and differences in contractile response magnitudes due to different sized veins (Klotz et al., 2006).

$$\text{Percent contraction} = [\text{sample response (Max-Min)}/\text{NE response (Max-Min)}]*100 \quad (1)$$

The min and the max response of the sample are the myographic readings (tension in smooth muscle, g) before and after addition of treatment, respectively. For dose response curve, a non-linear standard curve was established by plotting the normalized mean agonistic activity on smooth muscle against the concentration of ergotamine i.e., log (agonist) vs. response (equation 2).

$$Y=\text{Min} + (\text{Max-Min})/ (1+10^{((\text{LogEC50-X})*\text{Hill Slope})}) \quad (2)$$

The EC_{50} (effective concentration) is the concentration of agonist that gives a response half way between the minimum (low contractile response) and maximum (high contractile response), expressed in percent. Hill Slope describes the steepness of the curve.

To determine the ability of a polymer to reduce the contractile response, the NE-normalized percent contraction of smooth muscle to ETA was fitted against the polymer levels in the media i.e., log (inhibitor) vs. response (equation 3).

$$Y = \text{Max} + (\text{Max-Min})/ (1+10^{(X-\text{Log}IC_{50})}) \quad (3)$$

Maximum (Max) and minimum (Min) are plateaus in the units of the % contraction (NE Normalized) and IC_{50} (inhibitory concentration) is the concentration of polymer (mg) that is half way between maximum and minimum inhibition.

The isothermal adsorption efficiency of polymers to ergotamine tartrate in buffer media was determined using equation 4.

$$\text{Amount Adsorbed (moles}\cdot\ mg^{-1}) = (B_{max}*X)/ (K_d+X) + NS*X \quad (4)$$

Where, B_{max} is the maximum specific adsorption (moles· specific weight of polymer^{-1}), K_d is the equilibrium adsorption constant (mg of polymer) indicating amount of polymer needed to achieve a half-maximum adsorption at equilibrium and NS is the slope of nonspecific adsorption (moles· mg^{-2}).

The percent contractile responses of ETA after the 60-min incubation period were compared between the polymer types. The experimental model used was completely randomized design and data were analyzed using the mixed procedure of SAS (version 9.2; SAS Inst. Inc., Cary, NC) considering the effect of treatment as fixed effect with vein cross-sections serving as the experimental unit. Values were shown as mean ± SEM. For agonist or inhibitor effects on smooth muscle contraction, statistical analyses were performed by using GraphPad Prism Software, version 5.0 (USA). Non-normally distributed data analyses were conducted with non-parametric t-test followed by the Mann-Whitney test. Concentration–response curves were fitted by nonlinear regression using log (agonist) versus response and a nonlinear regression log (inhibitor) versus response was used for polymer evaluation. Differences between the means are declared significant for *P*-values lower than 0.05 (two-tailed test).

6.3. Results

6.3.1.Dose response

The contractile response of lateral saphenous vein cross sections to increasing concentrations of ETA was described by a sigmoidal relationship (R^2 = 0.95; Figure 6-1; Table 6-1) with the log concentration of ETA. The relationship was generally linear between 9.766E-08 and 6.250E-06 M, and the maximum contractile response of 88% was observed at and above 3.125E-06M. The effective concentration (EC_{50}) of ETA that produced 50 percent of the NE-normalized contractile response in terms of –log (ETA) was 6.66 ± 0.17 (2.26E-07M). The results were comparable to –log EC_{50} values of 6.48 ± 0.38 M obtained for mesenteric veins from steers fed endophyte free tall fescue seed when exposed to ETA (Egert et al., 2014). Although the range of ETA concentrations used to construct the dose response curve may have exceeded levels expected to be physiologically relevant, these concentrations were appropriate for achieving saturation of receptor-agonist interactions to allow for accurate calculation of the EC_{50}.

6.3.2.Validation studies

Results indicated that an incubation time period of 60 min reduced (P <0.001) contractile response compared to < 2 min period, when ETA solution was incubated either at 21 or 39 °C. However, there was no difference (P >0.05) in contractile response between incubation temperatures of 21 °C and 39 °C (Figure 6-2). The adsorption evaluation protocol included 60 minutes of incubation and 30 minutes of centrifugation. To reduce the time effect, an alternate protocol was used to evaluate adsorption: the incubation period of 60 min was reduced to 15 min and the centrifugation period of 30 min (25,000g, 39 °C) used to separate bound and free ETA was replaced with SPE filtration. Before implementing the new protocol, non-specific binding of ETA on SPE columns was evaluated. There was no difference (P =0.87) in myographic response between the ETA treatment (7.813E-07M) that was filtered through SPE column and the control (without SPE column filtration) (Figure 6-3). It was observed that the myographic response between the dose response study (no incubation, 39 °C) and adsorption study (15 min incubation, 39 °C) at similar concentrations differed (P <0.02) by 25 percent (Data not shown) indicating the effect of processing time on the myographic response.

6.3.3.Polymer evaluation: Myographic studies

Standard inhibition curves for MIP and NIP are shown in figure 6-4a. The smooth muscle contraction of saphenous vein decreased in a dose dependent fashion after ETA was exposed to increasing concentration of either polymer in the range from 0.625 to 10 mg, with 50 percent inhibitory concentrations of 0.511 and 0.729 mg for MIP and NIP, respectively. However, there was no difference (P = 0.163) in IC_{50} between MIP and NIP. Additionally, there was no difference in maximum (P = 0.96) and minimum (P = 0.26) inhibitory response between the polymers tested (Table 6-2). Furthermore, there was no difference (P = 0.24) in calculated area under the inhibitory response curve between MIP and NIP (Figure 6-4b).

6.3.4.Polymer evaluation: isothermal adsorption studies

The proportional amount of ETA adsorbed on the polymers with different inclusion rates of MIP and NIP are shown in figure 6-5 and the adsorption parameters derived from a one-site total binding curve are included in Table 6-3.

6.3.5. Biological implications of *in vitro* parameters

To relate the *in vitro* adsorption parameters to the *in vivo* biological responses with respect to the adsorption efficacy of the two polymers, the myographic contractile response was estimated for free concentration of ETA at equilibrium from isothermal adsorption studies. Myographic response data were estimated using the equation generated (Equation 5) from the dose response curve (Figure 6-1) and corrected by a factor of 0.75 obtained from validation studies for lowered myographic response subsequent to incubation at 39 °C for 15 min.

$$\text{NE normalized \% contraction} = 0.75\ (88.47 + (5.715 - 88.47)/(1 + 10^{\wedge(6.664 - \text{Free ETA concentration})})) \quad (5)$$

Pre-incubation of ergotamine in medium containing increasing concentrations of MIP or NIP reduced the contractile response in a dose dependent manner. To determine the biological implications of *in vitro* adsorption efficiencies, the *ex vivo* myographic response observed for treatments was related to adsorption efficiencies of polymers obtained from *in vitro* studies. The predicted contractile responses determined for free [ETA] in isothermal adsorption studies were regressed against the measured myographic response (Figure 6-6a and 6-6b, Table 6-4). Results showed that the contractile response for the polymers was predicted with a coefficient of determination of 0.82 and 0.87 for MIP an NIP, respectively. There was no difference in the slopes of the curve between MIP and NIP (0.98±0.06 and 0.92±0.05 for MIP and NIP, respectively), and the equations predicted the contractile response with no bias (P <0.01).

6.4. Discussion

An increased use of adsorbents in animal production signals the need for appropriate techniques to verify adsorption claims and product performance. Despite the availability of several *in vitro* models to characterize *in vitro* adsorption properties of adsorbents, a validated method to relate the *in vitro* adsorption parameters to *in vivo* biological responses is necessary. Myographic studies using blood vessels are useful in assessment of smooth muscle contraction in the presence of vasoactive compounds in the blood including a range of structurally different ergot alkaloids (Oliver et al., 1993; Klotz et al., 2006; Klotz et al., 2007; Foote et al., 2011a). Ergotamine and ergovaline have been shown to produce dose dependent smooth muscle contraction of blood vessels (Egert et al., 2014; Pesqueira et al., 2014). The current study evaluated the ability of synthetic polymer products to reduce the bioavailability of ETA thereby reducing vascular constriction of lateral saphenous in controlled conditions.

Ergotamine exists in two epimeric forms at the C-8 position and the extent of epimerization depends on duration of exposure to heat and light (Andrae et al., 2014). Studies have indicated that the R-form of the epimer is more biologically active than S-epimerized form (Crews, 2015). The measurement of blood vessel contraction is directly related to ETA concentration in the medium in the active isomeric form of ergotamine. When ergotamine is present in naturally contaminated fescue, they exist in both interconvertible epimers (R- and S-epimer) with an intermediate conformation (enol-intermediate) that is induced by variation in pH, exposure to strong light and temperature (Hafner et al., 2008; Andrae et al., 2014). The initial two validation studies evaluated the influence of incubation time and temperature on the vasoconstrictive response to ergotamine, and the third validation study confirmed that there was no non-specific binding to the SPE column that would influence the myographic response. Our studies showed a decrease in the myographic response by nearly 25 percent when ergotamine was incubated for 1 hour at 39 °C. The reduced contractile response could be due to isomerization to the less active form of the molecule, ergotaminine. Therefore, a correction factor was used to estimate the free ETA concentrations following incubation. Additionally, the ETA stock solution was prepared using DMSO to increase the solubility and reduce precipitation in buffer solution. DMSO was present in all the samples at same proportion.

The concentration range of ETA used in the dose response experiments was obtained from earlier observations showing that vasoconstriction occurred with a distinct sigmoidal response, when the agonist was added in cumulative fashion (Egert et al., 2014; Pesqueira et al., 2014). To avoid potential carryover effects in the present experiment, we used single doses of ETA at different concentrations on each blood vessel to generate the dose response. The vasoconstrictive effect is in part related to the fact that ETA can directly interact with biogenic amine receptors on smooth muscle, especially serotonergic (Dyer, 1993; Schöning et al., 2001) and adrenergic receptors (Görnemann et al., 2008). Similar to cumulative dosing, application of a single dose to each myographic tissue sample with increasing concentration of ETA across blood vessels also generated a sigmoidal response curve. Similar sigmoidal response curves were previously reported with ergot alkaloids in different tissues including saphenous vein (Pesqueira et al., 2014), mesenteric artery and vein (Egert et al., 2014) and ruminal artery and vein (Foote et al., 2011a).

The selection of the 75th percentile ETA concentration for polymer adsorption studies was based on the assumption that the samples exposed to polymer treatments will produce contractile responses in the lower part of the linear range of the dose response curve. The selected concentration of ETA corresponds to 0.45mg·kg^{-1} (ppm) of feed on dry matter basis that would correspond to natural contamination levels of ergovaline in endophyte infected tall fescue grass. Ergovaline is the predominant alkaloid in naturally contaminated tall fescue (Rottinghaus et al., 1991) and is found in the range of 0.5 to 5 mg·kg^{-1}.Studies have suggested that the ergotamine concentration in the contaminated forage and seeds are generally found at the rate of 0-12 and 15-40 percent of ergopeptides, respectively (Yates and Powell, 1988; Thom et al., 2013). Levels of total ergopeptides in surveys of rye have been reported at over 7 mg·kg^{-1} (Krska and Crews, 2008) with ergotamine as the major alkaloid. These studies suggest that ergotamine is comparatively greater in *Clavicep* infection (rye) while ergovaline is in greater concentrations in *Epichloë* infection (tall fescue grass).

Compound efficacies are commonly compared using the IC_{50} values, i.e., the inhibitory concentration of the compound that decreases the agonist response by 50 percent (Wolff et al., 2015; Serra et al., 2016). The *in vitro* isothermal adsorption study showed that the concentration of MIP and NIP required to reduce the bioavailability of ergotamine by 50 percent (K_d values) was 0.51 and 0.57 mg, respectively. While comparing the effect of these polymers in reducing saphenous vein smooth muscle contractions, the efficacy of polymers to reduce ergotamine-induced maximal response by 50 percent (IC_{50}) was 0.51 and 0.73 mg for MIP and NIP, respectively. Increasing the concentration of both polymers decreased the contractile response, but there was no difference between MIP and NIP in reducing the bioavailability of ETA. Although high affinity of imprinted polymer compared to non-imprinted polymer towards ergot alkaloids was noticed in other work (Lenain et al., 2012), our study revealed no difference in either affinity (K_d) or the IC_{50} values between imprinted and non-imprinted polymers.

Research have shown a direct relationship between the concentration of ETA and the intensity of the corresponding contractile response (Foote et al., 2011a; Egert et al., 2014). The prolonged and persistent contractile response is induced at low concentrations of ETA if the blood vessel is exposed for a longer period of time (Schöning et al., 2001; Pesqueira et al., 2014) due to strong interactions with serotonin receptor (Dyer, 1993; Klotz et al., 2007). This prolonged stimulation of serotonin receptors may result in receptor desensitization (Millan et

al., 2008) or receptor internalization (Tan et al., 2004) which may lead to inefficiency with treatments involving antagonistic mechanisms especially, receptor blockers. This sustained interaction of ETA with receptors also creates an opportunity for ergot alkaloid accumulation, which may exceed the rate of receptor recycling or turnover, causing greater impact on normal biological processes. Bioaccumulation in lateral saphenous vein after repetitive exposure to ergovaline *in vitro* has been suggested (Klotz et al., 2009). The potential for ergot alkaloid build-up in tissues of exposed animals could be one factor behind the persistent contraction and delay in vascular recovery after removal of animals from endophyte infected diets (Cunningham, 1949; Strickland et al., 2009). Additionally, ergopeptides including ergotamine and ergovaline have been shown to interact with serotonergic receptors and myenteric neurons in the gut affecting gut motility (Pan and Galligan, 1994; Briejer et al., 1997), and the baseline tone and inhibition of ruminal or reticular contractions (McLeay and Smith, 2006). Therefore, mitigation strategies that reduce ergot alkaloid bioavailability, especially with addition of ergot alkaloid-specific adsorbents can be considered more beneficial than measures attempting to treat the symptoms of ergot alkaloid toxicity.

The measured concentration of ergotamine remaining after adsorption (i.e., bioavailable concentration) was used to estimate the contractile response using the dose response curve (Equation 5). Others have estimated the biological significance of adsorbents by evaluating the excretion profile of ergot alkaloids in the presence of adsorbents (Adam et al., 2006) indicating lowered absorption of ergot alkaloids from the gastrointestinal tract. Evaluating the biological activity of absorbed ergotamine would indicate the concentration of ETA in body tissues, which in turn is an indirect measure of bioavailability of ETA and adsorption in the gut. Our results indicate that the parameters generated from *in vitro* studies to determine the efficacy of adsorbents can be extrapolated to their physiological significance in biological systems.

6.5. Conclusions

Ergotamine, along with other ergot alkaloids, can influence vascular smooth contraction of bovine lateral saphenous veins. This study indicated that a pre-myograph incubation with MIP or NIP at inclusion rate between 0.625 to 10 mg reduced the contractile response to ergotamine of the lateral saphenous veins in a dose dependent fashion. There were no indications that

imprinted polymers were more effective in reducing the contractile response compared to non-imprinted polymers. The *ex vivo* contractile response could be predicted from the *in vitro* adsorption data with more than 80 % accuracy indicating the physiological significance of the isothermal adsorption parameters. This study provides evidence that synthetic polymers can reduce the bioavailability of ergot alkaloids and the associated vascular contractile response induced by fescue toxicosis. However, further *in vivo* animal studies are required to assess the application of imprinted polymers as selective adsorbents of ergot alkaloids in feed.

Table 6-1. Dose response parameters for norepinephrine normalized contractile response of saphenous vein smooth muscle to increasing concentration of ergotamine.

Dose response parameters*	Mean	95% Confidence interval
Minimum contractile response, %	5.71	-13.93 to 25.36
Maximum contractile response, %	88.47	80.80 to 96.14
Log EC_{50}, -Log (ETA)	6.592	6.316 to 7.012
Hillslope, (% / -Log (ETA))	-1.386	-2.342 to -0.4295
Goodness of fit		
Degrees of freedom	9	
R square	0.95	

* Experiments were carried out with twelve increasing concentrations of ergotamine tartrate as a single dose exposure to each blood vessel in myograph chamber (n =10).
% contraction (NE normalized) = 88.47+ ((5.715-88.47)/ (1+10^ (6.664-intital [ETA])))

Table 6-2. Maximum and minimum inhibition and IC_{50} for nor-epinephrine normalized contractile response of saphenous vein exposed to ergotamine treated with MIP and NIP.

	Polymers			
Contractile response	MIP	NIP	SEM	*P*-value
At maximum inhibition, %	9.056	9.185	5.207	0.960
At minimum inhibition, %	88.004	69.665	29.871	0.265
IC_{50}, mg of polymer	0.511	0.729	0.306	0.163

Fifty percent inhibitory concentration (IC_{50}) values were calculated by a fitting concentration–response relation to a sigmoidal model of the form log (inhibitor) versus response (equation 3). The IC_{50} is the concentration of agonist in milligrams that gives a response half way between maximum and minimum inhibition.

Table 6-3. Adsorption parameters for ergotamine with each of two polymers in modified Krebs-Henseleit buffer media at 21 °C.

	MIP	NIP
B_{max} (Moles) [a]	3.274e -008 ± 2.391e-008	3.145e- ± 3.877e- 008
K_d (mg of polymer) [b]	0.5088 ± 0.108	0.567 ± 0.193
NS (Moles· mg^{-2}) [c]	-2.0553-010 ± 2.361e-010	-9.633e-011 ± 3.776e-010
N (sample size)	10	10
R^2	0.9267	0.8345

[a] B_{max} is the maximum specific adsorption of ETA in moles .

[b] K_d is the equilibrium adsorption constant which measures the level of polymer needed to achieve a half-maximum adsorption at equilibrium

[c] NS is the slope of nonspecific adsorption.

Table 6-4. Regression equation and fit parameters for the prediction of contractile response (Y) (NE-normalized) of saphenous vein smooth muscles by ergotamine (x) in the presence of MIP and NIP.

	Regression equation	R^2	*P*-value
MIP	Y = 0.98±0.06(x)+0.15±2.07	0.82	<0.01
NIP	Y = 0.92±0.05(x)-1.35±1.74	0.87	<0.01

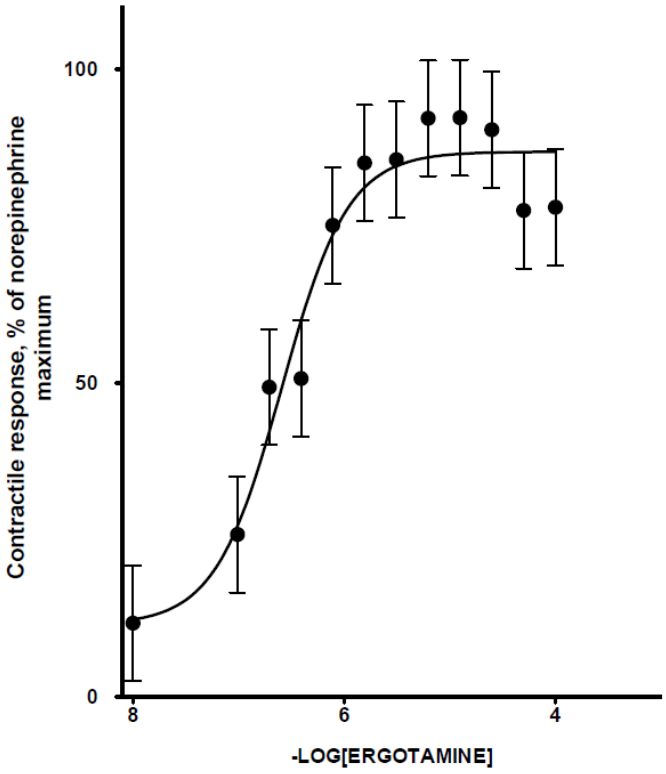


Figure 6-1. Norepinephrine normalized contractile response curve of smooth muscle (lateral saphenous vein) to increasing concentration of ergotamine. The points are means and the vertical bars show the SEM (n=10). % contraction (NE normalized) = 88.47+ ((5.715-88.47)/ (1+10^ (6.664-intital [ETA])))

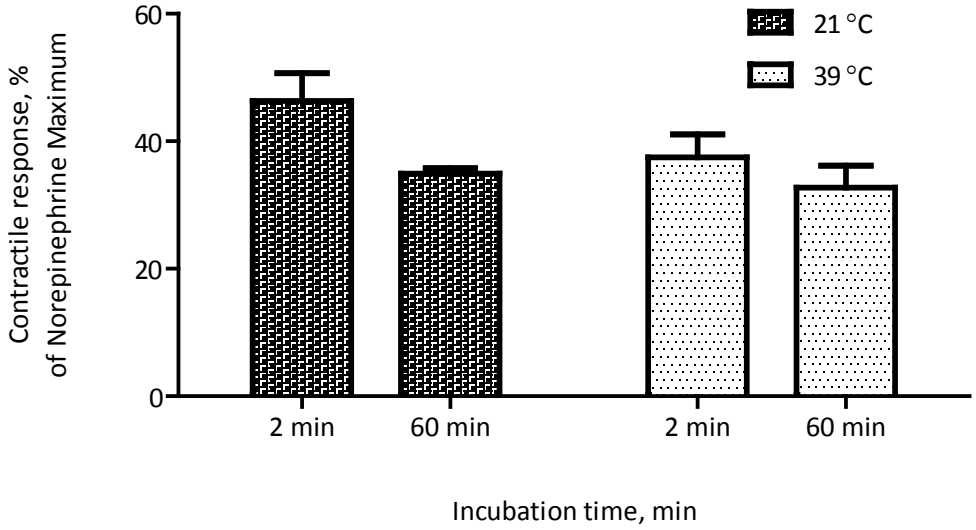


Figure 6-2. Effect of incubation temperature and length of incubation in buffer containing ergotamine (7.813E-07M) on the contractile response of saphenous vein. Each data point was the mean of 6 replications of blood vessel ± SEM.

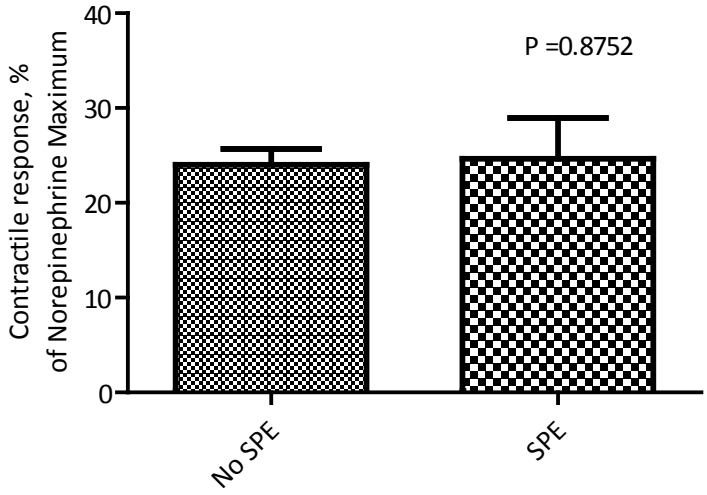


Figure 6-3. Contractile response of lateral saphenous vein exposed to ETA treatment (7.813E-07M) that are filtered through solid phase extraction columns at 21 °C (< 2min). Each data point represents mean of 6 replications of blood vessels ± SEM.

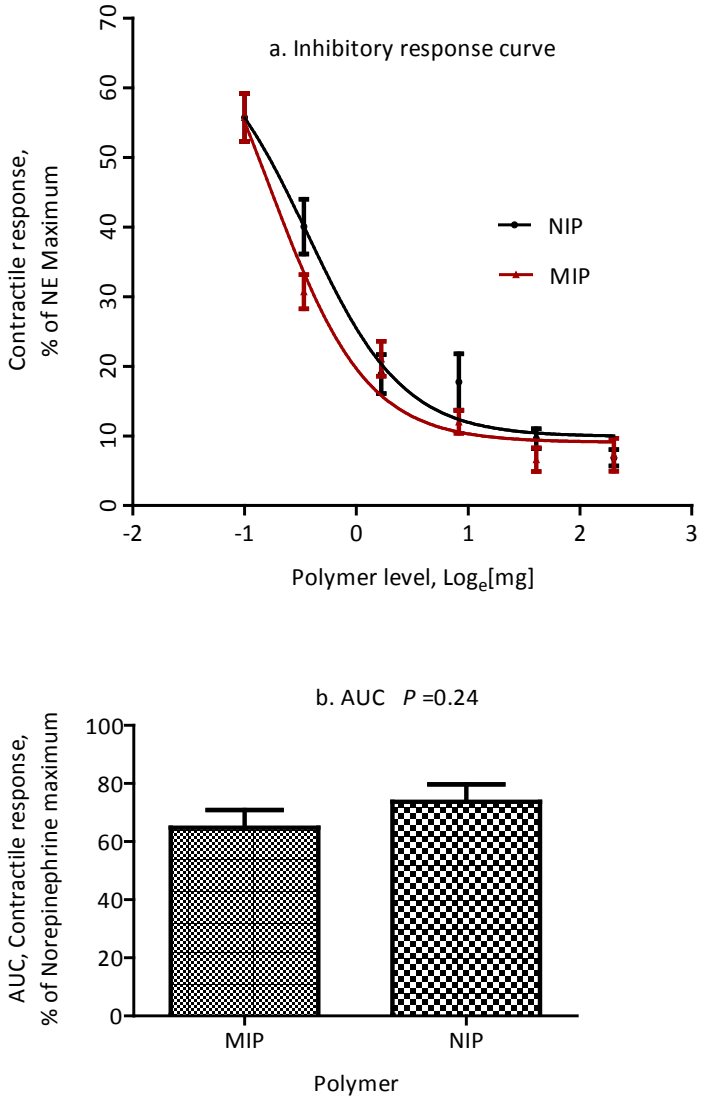


Figure 6-4. a) Effect of increasing dose of MIP and NIP on NE-normalized contractile response of saphenous vein induced by ergotamine (7.80E-06M). b) T-test analysis of area under the curve for comparison of MIP and NIP. Data are expressed as mean ± SEM (n=12).

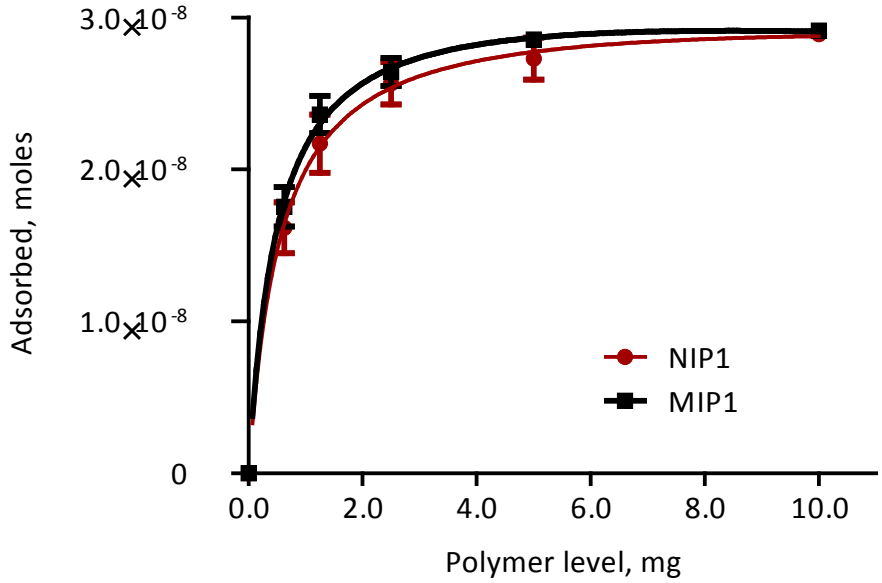


Figure 6-5. Ergotamine adsorption to polymers (MIP and NIP), in modified Krebs-Henseleit buffer media fitted to binding saturation curve with specific binding sites.

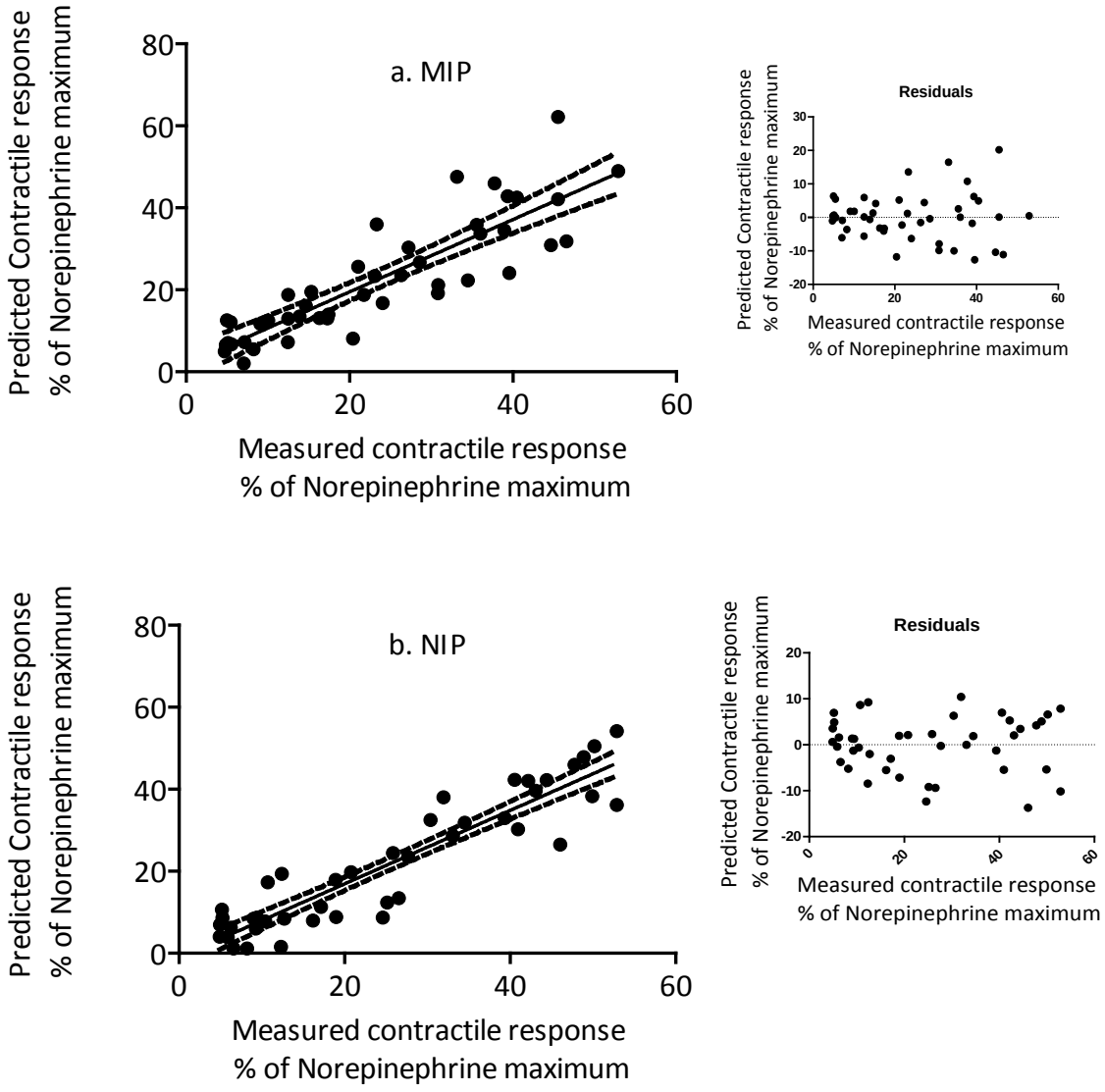


Figure 6-6. Regression plot between the measured and the predicted percent contractile response (NE-normalized) in the presence of increasing concentration of a) molecularly imprinted polymer and b) non-molecularly imprinted polymer. Dotted lines are 95% confidence interval. The residual are shown on the right side of the plot.

7. CONCLUSIONS AND IMPLICATIONS

Ergot alkaloids produced by the fescue endophyte fungus *Epichloë coenophiala* are known to produce complex biological responses in animals via agonistic and antagonistic interactions with biogenic amine receptors for serotonin, norepinephrine and dopamine. Strong and prolonged vasoconstriction is one of the mechanisms behind alkaloid induced toxicity. Among the two structural classes of ergot alkaloids, ergopeptides are known to produce more persistent dose dependent vasoconstriction than ergolines. Many of the secondary effects of toxicosis including altered thermoregulation, cardiovascular changes and reproductive problems have been well characterized. The yearly financial loss in the United States attributed to endophyte toxicity is estimated to have increased to billions of dollars. Mitigation strategies including dilution, use of novel endophyte, vaso-relaxatives and application of specific toxin adsorbents have been attempted. The goal of the research presented in this dissertation was to synthesize ergotamine templated molecularly imprinted polymers as specific adsorbents for ergot alkaloids and to evaluate the effect of imprinting on its adsorption properties towards ergot alkaloids. This work also evaluated the effect of polymers on *in vitro* fermentation and biological implications of *in vitro* adsorption studies.

In the first experiment, styrene based MIP and NIP that were synthesized using ETA as a representative template molecule were evaluated for adsorptive characteristics toward a variety of ergot alkaloids. Results suggested that functional groups including indole NH, carbonyl and hydroxyl groups present on the template molecule can contribute to physical interaction with polymers via hydrogen bonding while the resonance property of ring structures could contribute to π-stacking interactions. MIP was highly porous and had almost twice as much external surface area and pore volume compared to NIP. Freundlich isotherms indicated that both polymers had favorable adsorption but no difference was noted between MIP and NIP. However, MIP had better adsorption efficiency towards ergotamine in rumen fluid compared to NIP indicating some improvement in adsorption in a complex *in situ* environment. The imprinted polymers have properties conducive to be used as a sorbent in analytical extraction process to extract ergot alkaloids from complex media like rumen fluid which can be applied in sample preparation prior to HPLC or LC-MS/MS analysis. Even though styrene based polymers have been utilized in a variety of studies for imprinting, many studies with biological applications have

used methacrylate-based imprinted polymers. Imprinted polymers made from acrylate monomers have been evaluated as pH-responsive, slow drug delivery systems in the gut suggesting acceptability of polymethacrylates without adverse effects. Therefore, our subsequent experiments were targeted towards MAA-based ergotamine-imprinted polymers with an ultimate goal of utilizing them as an alkaloid adsorbent in animal feed to reduce bio-availability of ergot alkaloids in the gut.

In the second experiment, imprinted polymer was again synthesized, this time using methacrylic acid as the functional monomer. During pre-polymerization, non-covalent interactions like hydrogen bonding between methacrylate monomers and polar solvent insoluble template was favored in aprotic porogen (toluene). MIP had numerically better adsorption capacity and specificity towards ergot alkaloids compared to NIP. The presence of the ester carbonyl groups in the EGDMA crosslinker, hydroxyl group of HEMA and the carbonyl groups of the MAA may lead to the formation of additional hydrogen bonds with the hydroxyl groups of the ETA. Even though the adsorption coefficient of the MIP and NIP for the template did not differ in MacDougall's buffer, the imprinted polymer exhibited greater strength in binding to ergot alkaloids in the presence of feed components. Such polymeric material or organic alternatives could represent a novel strategy for new feed additives, specifically applied to animal production with the intent to mitigate the toxic effects of ergot alkaloids in livestock.

Subsequently, the effect of MAA-based imprinted polymers on *in vitro* ruminal fermentation was evaluated. Since the application of MIP as a toxin adsorbent in ruminant feed is unique, potential effects on ruminal microbial fermentation was of interest. Studies have shown toxicity of functional monomers like ethyl acrylate and methyl methacrylate in their monomeric form, but not in polymeric form. Although polymer degradation is unlikely in the rumen environment, their effect on ruminal fermentation in their polymeric form was also of interest. This study showed that polymer exposure at levels between 0.03 to 30mg per 500mg alfalfa DM, for 30 h at pH of 6.8, did not affect microbial fermentation. At very high levels (300 mg), small effects on fermentation pH were noted. At levels which would be reasonable to include in ruminant rations, these polymers had no detectable effect on *in vitro* fermentation suggesting absence of nonspecific binding to nutrients required for microbial growth. Even though the *in vitro* fermentation experiments mimic some of the complexities existing in the

rumen, it still falls short of representing the complete dynamics of the GIT. Therefore, additional *in vivo* studies are needed to ascertain the applicability of synthetic polymers as specific adsorbent materials in the presence of ergot alkaloids.

In the final experiment, to help validate the physiological significance of *in vitro* studies, *ex-vivo* myographic studies were conducted and the effect of polymer addition on bio-availability of ergotamine was evaluated. Pre-myograph incubation with MIP or NIP at inclusion rates between 0.625 to 10 mg reduced the contractile response to ergotamine in lateral saphenous vein in a dose dependent fashion. The *ex vivo* contractile response could be predicted from the *in vitro* adsorption data with the correlation coefficient of more than 80 percent. This study indicated that the biological implications could be predicted using the isothermal adsorption parameters with certain degree of confidence. This study also provided evidence that polymers have potential to reduce the bioavailability of ergot alkaloids and the associated contractile response often associated with fescue toxicosis. However, further animal studies are required to determine application of MAA-based polymers as selective adsorbents of ergot alkaloids in feed.

There are several reports in the literature indicating that imprinted polymers have better adsorption properties compared with non-imprinted polymers and different commercial adsorbents. In the present study, adsorption isotherms showed no difference between MIP and NIP in their adsorption properties, especially in aqueous media. The lack of difference between MIP and NIP could be due to incomplete template washing, which is critical for creating complimentary functional groups and cavities in imprinted polymers for rebinding. Incomplete washing of template could be a function of either strong binding via several types of non-covalent interactions between the template and the monomer during pre-polymerization or of large template size impeding template removal. However, even with lack of difference in aqueous media, MIP had better adsorption to template compared to NIP in the presence of feed components. MISPE studies also indicated that MAA-based MIP retained greater amounts of ergot alkaloids during washing compared to NIP. Stronger interactions of ergotamine with MIP in an MISPE column could be utilized for either extraction or purification in analytical chemistry.

It is possible that modifications to the imprinting approach could improve specificity of binding for MIP. Certain measures could be undertaken including the fine-tuning of the

synthesis approach, selection of monomers to increase the specificity for the target and modification of template removal procedures. UV initiated suspension polymerization is an alternative to thermal initiated bulk polymerization that has been reported to increase the specificity of imprinted polymers. Another approach in synthesizing polymer with several different ratios of monomer to crosslinkers is by combinatorial imprinting as proposed by Lanza and Sellergren (1999) and Takeuchi (1991) for MIPs targeted towards triazine and sulphonylurea, respectively. That approach involves the synthesis of a large number of polymers in HPLC vials (~50 mg) to determine an optimal MIP formulation for the template. The application of this technique has been aimed at finding appropriate monomers for a certain template or target analyte, and to find optimal monomer ratios and porogens to enhance molecular recognition and suppress nonspecific adsorption of analyte. The combinatorial approach provides an improved method over the conventional trial-and-error approach. However, one drawback is the limited range of methodologies available for the screening of those large number of MIPs. The use of rebinding studies is not feasible. To address this issue, Takeuchi (1991) developed a faster screening method that would allow evaluation of binding capacity by fluorescence measurements using a microplate reader.

There could be certain concerns with biocompatibility of styrene-based synthetic polymers if the application is to add in animal feed to bind ergot alkaloids. The concern is due to the fact that there are limited data on toxicity of styrene based polymers. With this regard, acrylate based polymers are more frequently tested in biomedical field and known to have low toxicity. Although poly-methacrylates have been more frequently used in applications like slow drug delivery in GIT and pH responsive release of drugs, use of these polymers as potential feed additives requires additional research. With regard to biocompatible polymers, studies have shown that many bio-based polymers such as chitosan, poly (lactic acid), poly (ε-caprolactone) and poly (hydroxybutyrate) are biodegradable and can be applied in industrial and agricultural fields.

Generally, chitosan is produced only by fungi or are commercially prepared by the chemical de-N-acetylation of chitin under alkaline conditions. Depending on the source of natural chitin and the conditions of its production, chitosan preparations can differ in size (average molecular weight; MW), degree of N-acetylation and other physicochemical properties

such as polydispersity (MW/MN), crystallinity or the pattern of acetylation. Basically, chitosans are linear polysaccharide consisting predominantly of unbranched chains of β-(1→4)-2-acetoamido-2-deoxy-D-glucose, synthesized from the deacetylation of chitin. They are insoluble in water (pH = 7) and common organic solvents, although they can be dissolved in specific solvents such as hexafluoro-2-propanol, N,N-dimethylacetamide or hexafluoroacetone. It has been considered as one of the most promising biopolymers for the development of advanced materials because of its low toxicity. Chitosan-based scaffolds have been shown to be biodegradable, non-immunogenic and biocompatible, and thus are widely used as therapeutic scaffolds for tissue engineering processes (Alves and Mano, 2008; Amaral et al., 2009; Pillai et al., 2009). Subcutaneous implantation of the chitosan microfibers demonstrated that implantation of rat muscle-derived stem cells containing chitosan microfibers induced lower host tissue responses with decreased macrophage accumulation (Kang et al., 2010). Chitosan can be modified by acylation, alkylation, etherification, esterification or halogenation, thereby providing derivatives with different structures and chemical properties that can be conducive to increase the adsorption properties.

Poor physical properties and high swelling degree of chitosan in aqueous systems limit its practical application. The glycosidic bonds of chitosan may be hydrolyzed leading to poor stability. In its crystalline form, chitosan is normally insoluble in aqueous solutions above pH 7; however, in dilute acids, the protonated free amino groups facilitate the solubility of the molecule. The pKa of primary amino groups depends closely on the degree of N-acetylation, so the solubility of chitosan is also dependent on the same degree of N-acetylation. Chemical cross-linking is conventionally used to overcome the aforementioned shortcomings (Mukoma et al., 2004). One of the most effective methods for enhancing the thermal and mechanical properties of biopolymers is by copolymerization with other monomers or polymers. Recently, ricinoleic acid, glyoxal (Yang et al., 2005), hydrocinnamic acid, polyethylene glycol, poly(ε-caprolactone) glutaraldehyde (Beppu et al., 2007) have been employed to prepare a series of bio-copolymers. The unique properties of chitosan, such as nontoxicity, biodegradability, biocompatibility and advantageous physical and mechanical performances of cross-linked chitosan, make chitosans a promising alternative to other functional monomers in molecularly imprinting technology.

The advantage of MIPs as adsorbents to reduce the bioavailability of ergot alkaloids compared to other absorbents are several. First, the inclusion rate compared to other adsorbents is very low. Compared to Yeast cell wall (YCW) based adsorbent, the adsorption efficacy of MIP and NIP was ten times higher, while comparing to clay based adsorbents, the efficiency was fifty times better. Second, No dilution effect of feed nutrients. Normal adsorbent inclusion rate recommended for yeast cell wall and clay based adsorbents is 1 and 5 kg/t, respectively. The inclusion rate of polymers to achieve same adsorption properties is 0.1 kg/t. Third, strong interaction between MIP and ETA could reduce the bioavailability much better than YCW or the clay based adsorbent. Finally, the effect of pH on adsorption properties are minimal, thereby withstanding the pH variation through the GIT

Additionally, even though MIP can be developed with a representative alkaloid as a template, the effectiveness in reducing the alkaloid bioavailability can vary in the presence of co-contamination. The pyrrolizidine alkaloids (lolines) and ergot alkaloids (ergopeptide and ergolines) are natural toxicants that coexist in endophyte-infected fescue. Toxicities of both loline alkaloids and ergopeptine alkaloids have been established in the last two decades. However, little is known about potential interactions among the various alkaloids with respect to the development of clinical signs of fescue toxicosis. Thus, adsorption of different alkaloids by MIPs in co-contaminated diets needs to be studied. Above all, animal studies with co-contaminated diets and mitigation through use of polymers is required before the potential value of dietary-supplemented polymers can be assessed.

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Education

1997-99 - Master of Veterinary Science, Department of Poultry Science. University of Agricultural Sciences, Bangalore, India. Research title: Ability of Esterified Glucomannan to reduce the toxicity of T-2 toxin in commercial layers".

1992-97 - Bachelor of Veterinary Science, University of Agricultural Sciences, Bangalore, India.

1990-92 - Pre-University college, St. Aloysius College, Mangalore, India.

Academics

2004 -QA/QC workshop. Presented "The importance of Mycotoxin and evaluation of mycotoxin adsorbents in adsorbing toxin and their mode of action" in China (Beijing), Thailand (Bangkok) and India (Bangalore).

2003 - Training on Gerhardt, Kjeldhal digestion and distillation equipment on principle, application, programming, operation, maintenance and safety procedures for the VAP 20, KB 20 and TUR/K

2001 - Completed "Beer 101" course for the beer hobbyist and small batch brewers

1986 - Passed 29th Annual UN Information Test, A wing of UNO

Awards/ Scholarship

2015: ASAS-ADSA-Presidential pick on e-poster

1998-99 World Poultry Science Association (WPSA) fellowship

1992-97 University of Agricultural Sciences Sports scholarship.

Oral Presentations

Kudupoje, M., V. Akay, and K. A. Dawson. 2003. Comparison of Commercial Mycotoxin Adsorbents for their Adsorption Kinetics with Aflatoxin B1 *in vitro*. The Second World Mycotoxin Forum, Noordwijk aan Zee, Netherlands. February 17-18, 2003

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Manuscripts in preparation

Synthesis, evaluation and characterization of a styrene based ergotamine imprinted polymer for potential use as an ergot alkaloid highly selective adsorbent

Characterization of hydroxyethyl methacrylic acid functionalized methacrylate based polymer imprinted with ergotamine template for ergot alkaloids adsorption

Effect of an imprinted-polymer ergot-alkaloid adsorbent on *in vitro* ruminal fermentation

Contractile response of bovine lateral saphenous vein to ergotamine tartrate exposed to different concentrations of molecularly imprinted polymers

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Patent

Yiannikouris A., Kwiatkowski S., Kudupoje M. August 26th 2010. Synthetic Mycotoxin Adsorbents and Methods of Making and Utilizing the Same. Application number US Ser. #12/870,664.